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(57) Abstract

The invention features a responsive RNA molecule which encodes, in one or more protein-coding regions, a polypeptide, and which includes a regulatory domain, a substrate region, and a ribosome recognition sequence. This responsive RNA molecule has an inhibitor region in the regulatory domain, which regulatory domain is complementary to both a substrate region of the responsive RNA molecule and to an anti-inhibitor region of a signal nucleic acid such that, in the absence of the signal nucleic acid, the inhibitor and substrate regions form a base-paired domain the formation of which reduces the level of translation of one of the protein-coding regions in the responsive RNA molecule compared to the level of translation of that one protein-coding region observed in the presence of the signal nucleic acid. The anti-inhibitor region of the signal nucleic acid is complementary in sequence to the inhibitor region of the responsive RNA molecule such that when the anti-inhibitor region is base-paired with the inhibitor region, translation of one protein-coding region of the responsive RNA is increased compared to the level of translation of that protein-coding region observed in the absence of the signal nucleic acid. The signal nucleic acid comprises part of the nucleic acid of a plant cell infecting organism.

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DESCRIPTION

VIRUS RESISTANT PLANTS CONTAINING INDUCIBLE CYTOTOXIC mRNAs

Background of the Invention

The invention relates to methods and compositions suitable for producing virus resistant plants.

One of the most important defense mechanisms in plants is the hypersensitive reaction. This occurs during an incompatible host pathogen (fungi, bacteria, virus, or nematode) interaction in which cellular changes take place that lead to cell death. Pathogenic organisms confined to such necrotic tissue quickly die or are restricted in their ability to replicate and spread an infection. Hypersensitive responses leading to necrotic lesions include a loss of permeability of cellular membranes, increased respiration, the accumulation and oxidation of phenolic compounds, and production of phytoalexins. Increased levels of specific phenolic compounds and induced phytoalexins are toxic to fungi and many bacterial and nematode pathogens. In virus diseases, the hypersensitive response results in so-called local lesions in which virus may survive in. low concentrations for a considerable time, although the virus is confined to the lesion.

Summary of the Invention

This invention features a ribozyme which acts to specifically kill plant cells infected with a specific virus. This in turn allows production of an artificial hypersensitive response in plants. This type of response can be readily constructed and applied to the inhibition of many viral infections. For example, an artificial hypersensitive response as a result of viral infection in a tobacco plant by tobacco mosaic virus (TMV) can be specifically targeted. The ribozyme is constructed in such a way that a signal sequence in the viral genome

stimulates the intracellular production of a toxin, e.g., an E. coli polypeptide toxic to plant cells. This creates a hypersensitive response in the plants by killing cells infected with a virus. The secondary structure of the 3' end of the TMV positive strand RNA genome has been very well characterized, and thus the determination of non-base paired regions as possible signal sequences is readily performed.

Specifically, the invention features an RNA molecule, termed a responsive RNA molecule which, when present in a plant cell, responds to the presence of other nucleic acids. By "responds" is meant that the responsive RNA molecule will be translated to form one or more polypeptides in the presence of certain nucleic acids (which can hybridize to the responsive RNA) and will not be significantly translated to form these polypeptides in the absence of such nucleic acids. Such a responsive RNA molecule will generally encode one or more polypeptide molecules, the production of which depends on translation of that responsive RNA molecule. Generally, translation of the responsive RNA molecule, and thus production of polypeptide, will not occur in any particular cell unless a specific nucleic acid, termed a signal nucleic acid, is also present within that cell.

A responsive RNA can be used to kill or injure specific cells within a population of cells. For example, a responsive RNA may encode a toxin molecule which is produced from the responsive RNA only when the responsive RNA molecule within a given cell is exposed to a signal nucleic acid indicative of a condition (e.g., infection with a harmful virus such as TMV) requiring that the cell be killed. More specifically, the responsive RNA molecule may encode a cytotoxic protein such as cholera toxin, diphtheria toxin, ricin and the hok, gef, RelF or flm gene products of E. coli, and translation of the responsive RNA molecule and production of cytotoxic protein occurs only when the responsive RNA molecule is present within a cell

which is infected with TMV. Here, an RNA molecule specific to TMV or a portion of the TMV RNA genome serves as the signal nucleic acid and interacts with the responsive RNA molecule to allow translation of the toxin-encoding sequences of the responsive RNA molecule.

A responsive RNA molecule is produced by designing a polypeptide-encoding RNA which, in the absence of a signal nucleic acid, has a structure which prevents translation. One type of responsive RNA molecule can fold to form a base-paired domain, e.g., which, when sufficiently stable, prevents translation by preventing the translational machinery of a cell from reading the nucleotide sequence of the RNA. A specific example of a responsive RNA molecule of this type has a domain which encodes the desired polypeptide (or "protein-coding region") and a regulatory domain (i.e., a domain which includes regulatory elements including an inhibitor region, inverted repeats and nucleation regions). The regulatory domain may be located anywhere in the responsive RNA molecule so long as the sequence of the elements of the regulatory domain are selected so as not to interfere with the activity of the coded polypeptide. The inhibitor region is complementary in sequence to both a substrate region (which can include portions of either the protein-coding region and/or a leader region which is the non-translated RNA 5' of the protein-coding region or portions of the RNA genome of an RNA virus) and to a region of the signal nucleic acid referred to as an anti-inhibitor region. In the absence of the signal nucleic acid, the inhibitory region of the responsive RNA molecule hybridizes to the substrate region of a responsive RNA molecule forming an intramolecular base-paired domain which prevents or reduces translation. When the signal nucleic acid is present, the anti-inhibitor region competes with the substrate region for binding to the inhibitor region. Formation of an intermolecular base-paired domain between the anti-inhibitor region of the signal nucleic acid and the

inhibitor region of the responsive RNA prevents formation of a base-paired region with the protein-coding region; under these circumstances the protein-coding region(s) can be translated.

A second type of responsive RNA molecule has an intervening sequence or "intron", the presence of which prevents translation of one or more "exons". Introns do not code for the desired polypeptides. Segments of the RNA which code for desired polypeptides are called "exons" as are non-coding sequences (e.g., the leader region, secretory signal sequences, poly(A) tails, and the like) that remain after the splicing reaction. This second type of responsive RNA molecule is designed so that it can undergo a splicing reaction under desired conditions (e.g., in the presence of a specific RNA molecule) which removes the intron and joins the two flanking portions of the RNA molecule, thus forming a molecule which is the proper template for the active polypeptide. It is the regulation of this splicing reaction which in turn regulates translation. This second type of responsive RNA molecule is similar to the first type of responsive RNA molecule in that it has an inhibitor region which is complementary in sequence to both the anti-inhibitor region of a signal nucleic acid and to a substrate region within the responsive RNA molecule. In this second type of responsive RNA, the substrate region is not necessarily part of an exon, but rather contains a region which is essential to the self-splicing reaction. When the substrate region is base-paired to the inhibitor region, the self-splicing reaction cannot occur, thus translation is prevented. In contrast, when a signal nucleic acid is present, its anti-inhibitor region hybridizes to the inhibitor region of the responsive RNA forming an intermolecular base-paired domain, which prevents intramolecular base-pairing between the inhibitor region and the substrate region. Under these circumstances, the substrate region is free to participate in the splicing

reaction, the intron is removed, and translation of properly joined exons can occur.

Thus, in a first aspect the invention features a responsive RNA molecule which encodes, in one or more protein-coding regions, a polypeptide, and which includes a regulatory domain, a substrate region, and a ribosome recognition sequence, e.g., a ribosome binding site, a translation initiation site, and all non-coding regions necessary for the translation of an RNA. This responsive RNA molecule has an inhibitor region in the regulatory domain which is complementary to both a substrate region of the responsive RNA molecule and to an anti-inhibitor region of a signal nucleic acid such that, in the absence of the signal nucleic acid, the inhibitor and substrate regions form a base-paired domain which reduces the level of translation of the responsive RNA molecule compared to that level observed in the presence of a signal nucleic acid. The signal RNA is chosen from one present only in plant cells which must be selectively killed, e.g., TMV genomic RNA or mRNA.

The "regulatory domain" is a region of the responsive RNA molecule which will regulate the level of translation of the responsive RNA molecule dependent upon the presence of the signal nucleic acid. The regulatory region includes the inhibitor region, inverted repeats and nucleation regions. A "ribosome recognition sequence" is a region of an RNA molecule that is required in order for translation to begin at a given initiation codon (typically AUG). Such a site is recognized by a ribosome and bound by the ribosome prior to the initiation of translation of the RNA. In procaryotes, the ribosome recognition sequence is a ribosome binding site and includes a purine-rich sequence centered about 10 nucleotides 5' to the initiation codon (Shine and Dalgarno, Proc. Natl. Acad. Sci. USA 71:1342, 1974). For eucaryotes, the sequence A/G NNAUGG described by Kozak (Kozak, J. Cell Biol. 108:229, 1989) is the minimal

ribosome recognition sequence required for initiation of translation. This sequence includes the AUG initiation codon.

The "signal nucleic acid" is a nucleic acid (e.g., a viral RNA) which is indicative of a condition under which it is desirable to produce the polypeptide encoded by the responsive RNA molecule.

A "base-paired" domain is a region over which the nucleotides of two regions of nucleic acid are hydrogen bonded to each other. The term includes bonding of less than all contiguous nucleotides of such regions.

The "substrate region" is a region of the responsive RNA molecule which when base-paired reduces the level of translation of one or more of the protein-coding regions in the responsive RNA molecule.

The "inhibitor region" is a region of the responsive RNA molecule which when base-paired to the substrate region reduces the level of translation of one or more protein-coding regions in the respor..ive RNA molecule.

The "anti-inhibitor region" is a region of the signal nucleic acid which when base-paired to the inhibitory region increases the level of translation of one or more protein-coding regions of the responsive RNA molecule compared to that observed in the absence of the signal nucleic acid molecule. These three regions interact to regulate the level of translation of the responsive RNA molecule and are selected to insure appropriate levels of polypeptide production dependent upon the presence of the signal nucleic acid.

An "indicator gene" includes a coding region whose expression can be easily identified. For example, the genes encoding luciferase, β-glucuronidase, or chloramphenicol acetyltransferase.

By "appropriate level" is meant that in the absence of the signal nucleic acid the level of polypeptide is sufficiently low to have little or no effect on the physiology of the cell, and in the presence of the signal

nucleic acid the level of polypeptide is sufficiently high to reduce viability of the cell. The level of translation of the responsive RNA can be determined by standard procedures. Generally, a low level of translation is one in which less than 0.1% of the polypeptide produced by a cell is polypeptide encoded by the responsive RNA molecule.

In preferred embodiments, the substrate region is part of an exon or a leader region or overlaps the junction between the two (which includes the ribosome recognition sequence, and the initiation codon), or includes a region necessary for the self-splicing reaction.

In eucaryotic cells, the 40S subunit of the eucaryotic ribosome binds at the 5'-end of a capped mRNA and "scans" down the message in search of the first initiation codon (see generally Kozak, J. Cell. Biol. 108:229, 1989). In this process, all but extremely stable hybrids (i.e., those having a free energy of formation of <-50 kcal/mol) are unwound and scanned through (Kozak, Proc. Natl. Acad. Sci. USA 83:2850, 1986). Thus, to inhibit scanning of the 40S subunit to the translation initiation site, the inhibitor region must generally form an extensive hybrid with the substrate region (which may include the ribosome recognition sequence and/or the initiation codon) in which the base-paired region has a free energy of formation that is -50 kcal/mol or lower. Thus, it is preferred that the inhibitor region be located downstream (3') of the ribosome recognition sequence (in the exon or perhaps nearer the 3' end of the message) so that the interaction between the inhibitor region and the anti-inhibitor signal RNA (which would have a similar if not lower free energy of formation) would not also prevent movement of the 40S ribosomal subunit to the initiation site (see, Figs. 1F, 1G, and 1H). Accordingly, in a plant eucaryotic system, having the self-splicing intron interrupt the protein-coding region is preferred.

As used herein an "intron" is a domain of the responsive RNA molecule which is separate from the exons. Preferably the intron is an RNA molecule having catalytic activity including RNA cleavage and ligation activity. It is preferred that such an intron be able to self splice and thus is chosen from a group I or group II intron, such as that present in Tetrahymena thermophila.

In more preferred embodiments, the responsive RNA molecule is purified, and the responsive RNA encodes a polypeptide which modifies cell viability, cell proliferation, transcription of DNA, translation of RNA, or replication of DNA, e.g., the responsive RNA molecule encodes a polypeptide which has diphtheria toxin activity or ribonuclease activity.

"Purified RNA" is RNA isolated from one or more components of the environment in which it naturally occurs. For example, the RNA is present in a cell in which it does not naturally occur as is the case with foreign genes expressed in transgenic plants. Preferably it is provided as a homogeneous solution of nucleic acid.

In other preferred embodiments, the substrate region includes the 5'-splice junction of the intron; the intron reduces the level of translation of the exons compared to the level of translation in the absence of the intron; the intron is located between the ribosome recognition sequence and a 5'-most exon or between two exons. Even more preferably, the intron overlaps at its 5'-end a 5'-splice junction, and at its 3'-end a 3'-splice junction; the intron catalyzes two cleavage reactions, one within the 5'-splice junction and one within the 3'-splice junction; the intron is a self-splicing intron; the substrate region includes the 5'-splice junction; and the inhibitor region interferes with the cleavage reaction within the 5'-splice junction.

A "5'-splice junction" refers to the sequence overlapping or abutting the 5'-end of an intron which is required for a splicing reaction. A "3'-splice junction"

refers to the sequence at the 3'-end of an intron which is required for a splicing reaction. Such splice junctions overlap the ends of a self-splicing intron such as those bordering the intervening sequence of Tetrahymena thermophila.

A "self-splicing intron" is a piece of RNA which contains all of the sequences required except for the necessary abutting splice junction sequences for the intron to excise itself from a larger piece of RNA and to join the two pieces of RNA that flanked the intron prior to the excision reaction. That is, the intron is able to cleave and ligate two portions of an RNA molecule.

In yet more preferred embodiments, the signal nucleic acid is single stranded, e.g., it is viral RNA.

Examples of responsive RNA include Tetrahymena RNA which has been modified, for example, by nucleotide changes at positions -14, -19, -21, -22, -23 and/or -24 relative to the 5'-splice site.

In a related aspect the invention features a method for interfering with the growth of a cell harboring a signal nucleic acid by introducing a responsive RNA molecule as described above into the cell.

In another related aspect, the invention features a DNA molecule encoding the above responsive RNA molecules.

This invention is based upon the non-natural use of the hypersensitive response with RNA self-splicing dependent on the presence of specific RNA sequences. Ribozymes designed to undergo self-processing when hybridized to unique viral sequences offer a highly selective switch which will block translation of a toxic peptide until the plant is infected with a specific virus. Transgenic plants encoding such RNA will not produce a foreign or lethal protein until it is needed to combat a viral infection. Even when the toxic polypeptide in production is limited to the cells infected by a specific virus, and the death of infected cells will terminate production of the toxic protein. This application of RNA

processing technology significantly enhances the natural defense mechanism of the plant, and does not produce high levels of a foreign protein. Only low levels of the self-splicing RNA coding for a toxin are required, and as a result, little of the plant's resources are needed to produce an antiviral response.

Transgenic plants can be produced which are resistant to viral infections, and thus will have less crop damage and provide higher yields. This invention can be applied to all plant viral systems in which the viral genetic sequences are known or deciphered, and the infected plant can be transformed.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings are first briefly described.

Brief Description of the Drawings

Figs. 1, 1A and 1B are schematic drawings of a responsive RNA molecule. The thin line represents the leader region, the thick line represents a protein-coding region, a series of short vertical lines indicates a base-paired domain, and the boxes above and below these lines indicate various features of the RNA. Specifically, in Fig. 1A the responsive RNA is drawn so as to depict intramolecular base-pairing which prevents translation; and in Fig. 1B the responsive RNA molecule is depicted as hybridized to a signal nucleic acid.

Fig. 1C depicts a second variation of this type of responsive RNA molecule; in Fig. 1D the responsive RNA molecule is drawn to depict the intramolecular base-pairing that prevents translation; and in Fig. 1E the responsive RNA molecule is hybridized to a signal nucleic acid.

Fig. 1F depicts a third variation of a responsive RNA molecule; in Fig. 1G this responsive RNA molecule is drawn to show the intramolecular base-pairing which prevents translation; and in Fig. 1H the responsive RNA molecule is hybridized to a signal nucleic acid.

Figs. 2, 2A, 2B, and 2C are schematic drawings of a responsive RNA molecule which includes a self-splicing intron. The thin line represents a leader region, the broken line represents a self-splicing intron, the thick line represents an exon, a series of short vertical lines indicates a base-paired domain, and the boxes above and below these lines represent various features of the RNA. Specifically, in Fig. 2A the responsive RNA molecule is drawn so as to depict the intramolecular base-pairing which prevents self-splicing; in Fig. 2B the responsive RNA molecule is depicted as hybridized to a signal nucleic acid; and Fig. 2C depicts the spliced molecule produced by the self-splicing reaction.

Figs. 2D, 2E, 2F and 2G depict a variation of the type of responsive RNA molecule shown in Figs. 2-2C. In Fig. 2D, a self-splicing intron separates the polypeptide-coding sequence in the responsive RNA molecule; in Fig. 2E, the responsive RNA molecule is drawn to depict the intramolecular base-pairing which prevents self-splicing; in Fig. 2F, the responsive RNA molecule is hybridized to a signal nucleic acid; and in Fig. 2G, the spliced molecule produced by the self-splicing reaction is depicted.

Fig. 3 depicts P(1) and P(-1) stem-loop structures at or just upstream of the 5' exon-intervening sequence (IVS) junction of <u>Tetrahymena thermophila</u>. The IVS (uppercase) contains the internal guide sequence (boxed) which can hybridize with the end of the 5' exon (lowercase) to form the P(1) stem-loop, the conformation required at the 5'-splice site (shown by filled-in triangle) for self-splicing. The alternative structure P(-1), which does not support self-splicing, is formed by hybridization between

a portion of the P(1) stem (boldface) with an upstream 5' exon sequence (overlined). The sequence shown at the top is that for RNA from the parent plasmid pTETBLU. The lower three RNA structures represent modified P(-1) stem-loops from three mutant plasmids that were made by sequence changes (shaded) in the 5' exon. Calculated free energies at 37°C for each of these structures are given.

Fig. 4 is a copy of a photograph of a polyacrylamide gel showing the results of in vitro transcription reactions carried out in the presence of [α^{32}P]CTP using the parent plasmid (pTETBLU) and the three splicing mutants (pTET14, pTET1419, pTET21-24) as templates. Each set of three lanes represents the transcription products before (0) and after (15 or 60 min) the change to splicing conditions. The template used is given above each set of lanes, and the restriction enzyme used to linearize the template is shown at the top. For this and the following two figures, FL denotes the full-length precursor RNA and LE indicates ligated exons. The positions of linear IVS RNA (L-IVS) and circular IVS RNA (C-IVS) are also indicated, as are the shortened forms of L-IVS in which 15 or 19 nt have been removed from the 5' end by the circularization reaction (L-15 and L-19, respectively). An asterisk denotes an RNA thought to be the product of 3'-splice site hydrolysis (i.e., a 5' exon-IVS fragment). An as yet unidentified small RNA product is also indicated (<).

Fig. 5 is a copy of a photograph of a polyacrylamide gel showing the results of experiments in which gel-purified pTET1419 RNA was incubated under splicing conditions in the absence (0) or presence of the given concentrations of either of two signal RNAs (4S or 4S3) for 15 or 60 min. The resultant products were analyzed on a 4% denaturing polyacrylamide gel and are indicated in Fig. 5.

Fig. 6 is a copy of a photograph of a polyacrylamide gel showing the results of experiments in which gel-

purified pTETBLU RNA or pTET21-24 RNA (10 nM) was incubated in splicing buffer at 4 or 37°C. Where indicated, Mg^{2+} was added to 5 mM to initiate the splicing reaction. For pTET21-24, splicing was initiated in the absence or presence of either of two signal RNAs specific for the pTET21-24 sequence (8S4 or 12S). When present, the concentration of the signal RNA is 1 μM. The resulting products, analyzed on a 4% denaturing polyacrylamide gel, are labeled as in Fig. 4. Templates used for transcription were linearized with either *Eco*RI or *Bam*HI as indicated at the top. An additional product seen when the *Eco*RI-runoff precursor is incubated under splicing conditions in the presence of signal RNA is indicated with a dot. A short RNA product (<) seen when pTET21-24 is incubated under splicing conditions in the absence of a signal RNA is marked with an arrowhead. This same RNA product is also visualized in Fig. 4.

Transgenic Plants

Responsive RNA molecules, *e.g.*, ribozymes can be developed which undergo a self-splicing reaction when a target sequence in the RNA is base paired to an RNA signal sequence. This RNA enables the signal sequence induced release of intron sequences inhibiting the correct translation of a toxic polypeptide. Signal sequence induced RNA splicing can thus be used to selectively express a toxic polypeptide. This type of RNA or "killer ribozyme" is useful in the selective death of specific plant cells. Such ribozymes can be introduced into plants on standard vectors as DNA encoding the desired ribozyme.

The "killer ribozyme" provides the opportunity to produce an artificial hypersensitive response in plants. The "killer ribozyme" is constructed in such a way that a signal sequence in viral transcripts or the viral RNA genome stimulates the intracellular production of an *E. coli* polypeptide toxic to plant cells. This creates a hypersensitive response in transgenic plants by killing

cells infected with a virus. When a hypersensitive response is induced by viral infection, either a necrotic lesion will form, or if the response is extremely efficient the single infected cell will die before limited spread of infection to adjacent cells.

Thus, transgenic plants can be produced which mimic a hypersensitive response as a result of viral infection. This will result in inhibition of the spread and replication of virus in plants and a mode of producing viral resistant plants.

In order to design such a ribozyme, different signal sequences, non-signal induced splicing of the ribozyme, and the toxicity of the appropriate polypeptide can be assayed in, e.g., a tobacco protoplast system. Protoplasts can be induced to take up both expression vectors containing the ribozyme construct and viral particles. Instead of the toxic peptide sequences, the ribozyme may be constructed with an indicator gene which when expressed will be translated after signal sequences (viral genomic RNA) induce splicing of the ribozyme. This will provide the capacity to easily test different signal sequences and determine the degree to which non-signal induced splicing occurs.

Information derived from the protoplast assays can be used in developing "killer ribozymes" optimized for signal sequence and low nonsignal induced splicing. Utilizing this information, constructs can be developed for the production of transgenic tobacco plants, which respond to TMV infection with an artificial hypersensitive response.

Responsive RNA Molecules

Responsive RNA molecules are generally described above. Below are presented specific examples to illustrate these molecules to those of ordinary skill in the art. These examples are not limiting to this invention.

Example 1: Responsive RNA molecules without introns

A first type of responsive RNA molecule is illustrated in Fig. 1. One portion of this molecule, the protein-coding region encodes a polypeptide whose production is desired only in the presence of a signal nucleic acid. Another portion of the molecule, the regulatory domain, includes an inhibitor region which is complementary in sequence to a substrate region within the protein-coding region. The inhibitor region can base pair with the substrate region to form a base-paired domain which blocks translation of the protein-coding region. The substrate region can be a part of the protein-coding region, part of the leader region, or overlap the junction between the two.

Referring to Fig. 1, responsive RNA molecule 10 has a 5'-end 12, and a 3'-end 14. Adjacent to 5'-end 12 is a leader region 26 and regulatory domain 16; adjacent to 3'-end 14 is a protein-coding region 18. Within regulatory domain 16 is an inhibitor region 20; within protein-coding region 18 is a substrate region 22. At the 5' of protein-coding region 18 is a ribosome recognition sequence 21 and an initiation codon 23.

Referring to Fig. 1A, inhibitor region 20 hybridizes to substrate region 22 to form a base-paired domain 28. Such base-pairing within responsive RNA molecule 10 inhibits translation of the protein-coding region of the responsive RNA molecule.

The inhibition of translation is relieved by the presence of a signal nucleic acid, a region of which, referred to as the anti-inhibitor, is complementary to the inhibitor region of the responsive RNA. The anti-inhibitor region of the signal nucleic acid competes with the substrate region of the responsive RNA molecule for hybridization (base pairing) with the inhibitor region of the responsive RNA molecule. Under these circumstances there is no base pair formation with the substrate region,

translation occurs and the desired polypeptide is produced.

For example, referring to Fig. 1B, signal nucleic acid 30 has a 3'-end 32, a 5'-end 34, and an anti-inhibitor region 36 complementary in sequence to inhibitor region 20 of responsive RNA molecule 10. Hybridization of anti-inhibitor region 36 with inhibitor region 20 forms base-paired domain 38 and prevents hybridization of inhibitor region 20 to substrate region 22. Under these circumstances, translation of protein-coding region 18 occurs.

In a variation of this type of responsive RNA molecule, the substrate region is not entirely contained within the protein-coding region but extends upstream of the protein-coding region into the leader region. Specifically, the responsive RNA molecule depicted in Fig. 1C has a substrate region 22 which includes the ribosome recognition sequence 21 and the initiation codon 23. Referring to Fig. 1D, substrate region 22 base-pairs to inhibitor region 20 forming intramolecular base-paired region 28. In a procaryotic system, this configuration physically blocks a ribosome from interacting with the ribosome binding site and the initiation site, and translation is inhibited. Referring to Fig. 1E, the anti-inhibitor region 36 of the signal nucleic acid 30 is hybridized to the inhibitor region 20 to form base-paired region 38. In this configuration, a procaryotic ribosome initiates translation and the desired polypeptide is produced.

In another variation of this type of responsive RNA molecule, the inhibitor region is located downstream of the substrate region. The inhibitor region can be within the protein-coding region itself, as diagrammed in this figure, or located in a region 3' of the protein-coding region. In Fig. 1F, the responsive RNA molecule is depicted as having a substrate region 22 that includes ribosome recognition sequence 21 and initiation codon 23

and has an inhibitor region 20 located 3' of the substrate region. Referring to Fig. 1G, the inhibitor region, 20, base-pairs with the substrate region 22 forming intra-molecular base-paired region 28. In this configuration, a scanning eucaryotic ribosomal subunit cannot invade or bind to the base-paired domain to initiate translation provided this basepairing interaction is sufficiently strong. In Fig. 1H, the anti-inhibitor region 36 of signal nucleic acid 30 is hybridized to inhibitor region 20 forming base-paired region 38. A eucaryotic ribosome can scan to the proper initiation codon (provided there are no other upstream initiation codons) and initiate translation. Translation of the polypeptide occurs, with disruption of base-paired region 38 by the translating ribosome.

Since the inhibitor region of the responsive RNA must be complementary to both the substrate region of the responsive RNA, and the anti-inhibitor region of the target nucleic acid, the sequences of these three regions must be chosen to allow suitable regulation of translation of the responsive RNA. This does not mean that the sequence of the substrate region must be identical to the sequence of the anti-inhibitor region. Neither of the two base-paired domains which can form need to be perfectly base-paired (i.e., all contiguous bases along the domains are base-paired), nor do they have to be the same length. There is flexibility in the selection of the anti-inhibitor region so long as the region is specific enough to indicate when translation must occur. For example, if the signal to which the responsive RNA responds is the presence of TMV genome or transcripts within a cell, any specific nucleic acid sequence of TMV could be chosen, and of course, one is limited in selecting a nucleic acid sequence present in TMV. The sequence of the substrate region is chosen to create a responsive RNA molecule which produces a biologically active polypeptide. Since the substrate region may include portions of a protein-coding

region, any modification of its sequence must preserve a significant amount of the activity of the encoded polypeptide. The degeneracy of the genetic code allows for changes in the sequence of the protein-coding region which do not affect the sequence of the encoded polypeptide. Because guanosine can base-pair with uridine as well as with cytosine there is additional flexibility in the sequences which can be used. In addition, since conservative amino acid changes at one or more positions in proteins often do not eliminate activity of the protein the number of useful sequences is increased substantially.

The base-paired domain formed by hybridization of the inhibitor region to the substrate region must be stable enough so that it will not be disrupted by nucleic acids other than the signal nucleic acid, which may also be present within the cell. For example, if the inhibitor region and the substrate region are complementary over only four contiguous nucleotides, any single stranded nucleic acid that includes that four base sequence could compete with the substrate region for hybridization to the inhibitor region, and if the nucleic acid including this sequence was present at a high enough concentration inhibition of translation would be relieved. Generally, the base-paired domain formed by the hybridization of the substrate region to the inhibitor region should include at least 12, and preferably 15, contiguous nucleotides in order for the molecule to respond to only the signal nucleic acid.

The responsive RNA molecule can include a region that will allow the signal nucleic acid to more readily hybridize to the inhibitor region. This additional region is called a nucleation region and consists of a number of nucleotides immediately adjacent to the inhibitor region and complementary to the sequence of the signal nucleic acid such that the nucleation region and the inhibitor together form a region of extended complementarity with the signal nucleic acid. The nucleation region provides

a single stranded region that is readily available for hybridization to the signal nucleic acid. Base-pair formation over this region will tend to favor displacement of the substrate region from the inhibitor region by positioning the anti-inhibitor region correctly for hybridization to the inhibitor region. In addition, such a nucleation region will increase the stability of the base-paired region formed with a signal nucleic acid.

The regulatory domain may also include a region that will disfavor hybridization of non-specific nucleic acids (i.e., nucleic acids other than the signal nucleic acid) to the region immediately adjacent to the inhibitor domain. This region is referred to as an inverted repeat and can fold to form a hairpin structure.

The detailed nature of the inhibitor region, the substrate region, and the anti-inhibitor region will depend, in part, on the extent that translation is to be regulated. The more stable the intramolecular base-paired domain formed by hybridization of the inhibitor region to the substrate region, the more translation will be inhibited. For RNA-RNA duplexes, the stability of a base-paired domain depends on the number of nucleotides actually base-paired within a contiguous region of nucleotides, the number of mismatches within a generally base-paired domain, and the nucleotide composition of the base-paired domain. Intramolecular base-pair formation depends on the distance between the two regions to be base-paired. For example, when there are too few nucleotides between the two regions, torsional-type constraints can prevent base pair formation. Those in the art are well aware of how these parameters can be adjusted in order to make a more or less stable base-paired domain. The stability of the intramolecular base-paired domain can be adjusted dependent upon the level of translation that is desired at any given level of signal nucleic acid. The level of translation depends on the proportion of responsive RNA molecules in which the inhibitor region is

hybridized to the substrate region. This proportion, in the presence of the signal nucleic acid, depends on the proportion of the responsive RNA molecules in which the inhibitor region is hybridized to the anti-inhibitor region of the signal nucleic acid. Those in the art will appreciate that the amount of each duplex which forms depends on the relative stability of the two duplexes as well as the amount of signal nucleic acid and responsive RNA present in a given cell. If a highly toxic molecule is encoded by the responsive RNA then a high degree of regulation is required. For example, if the active subunit of cholera toxin is encoded, only a few molecules are required to kill a cell. In this case translation must be completely inhibited in the absence of signal nucleic acid. This is best insured by having almost complete complementarity of the substrate and the inhibitor regions, e.g., 85% complementarity of a 20 nucleotide region. Expression occurs only when a highly complementary signal RNA is present, having e.g., 100% complementarity to the inhibitor region over a 25 nucleotide region.

The inhibitor region may be on the 5'-side or the 3'-side of the protein-coding region or within the protein-coding region itself. If the responsive RNA molecule is subject to exonucleolytic degradation, this should be taken into account when designing the molecule. Thus, if the molecule is degraded beginning at the 3'-end it would be best to locate the inhibitor region at the 5'-end of the molecule in order to prevent formation of a molecule containing all of the sequences required for translation but lacking an inhibitor region.

Example 2: Responsive RNA molecules with self-splicing introns

A second type of responsive RNA molecule includes a self-splicing intron which prevents production of the desired polypeptide. The intron can be removed by a

splicing reaction, and the spliced molecule serves as a template for the production of the desired polypeptide. A signal nucleic acid regulates translation of this type of responsive RNA molecule, but the regulation is achieved indirectly by using the signal nucleic acid to regulate the splicing reaction. In order for this type of regulation to work the responsive RNA molecule must, in the absence of the signal nucleic acid, fold so as to form an intramolecular base-paired domain which prevents splicing. In the presence of the signal nucleic acid an alternative intermolecular base-paired domain forms and splicing occurs.

An example of this second type of responsive molecule is illustrated in Fig. 2. This molecule has an intron located between the ribosome recognition sequence and the initiation codon of a single protein-coding region which encodes a desired polypeptide. This intron prevents translation because it places the ribosome recognition sequence too far away from the initiation codon. In this example, the intron is a self-splicing intron derived from the pre-rRNA of Tetrahymena. Introns of this type can fold into a structure which causes two cleavage reactions, one on either side of the intron, and a ligation reaction which joins the portions of the RNA molecule flanking the intron. An essential step in the self-splicing of such introns is hybridization of a region of the intron, referred to as the 5'-splice junction, to a second region of the intron, referred to as an internal guide sequence. Thus, one way the self-splicing activity of the intron can be regulated is by preventing hybridization of the 5'-splice junction to the internal guide sequence. The responsive RNA molecule depicted in Fig. 2 has a regulatory domain which is distinct from the intron and the protein-coding region. This regulatory domain has an inhibitor region which is complementary to the substrate region which in this molecule includes the 5'-splice junction of the self-splicing intron. Intramolecular base

pair formation between the inhibitor region and the substrate region prevents hybridization of the 5'-splice junction to the internal guide sequence, and splicing is prevented. This responsive RNA molecule is designed so that the inhibitor region is also complementary to the anti-inhibitor region of the signal nucleic acid. Thus, in the presence of the signal nucleic acid, the inhibitor region hybridizes to the anti-inhibitor region freeing the 5'-splice junction for participation in the self-splicing reaction.

Referring to Fig. 2, responsive RNA molecule 40 has a 5'-end 42, and a 3'-end 44. Adjacent to 5'-end 42 is a leader region 49 adjacent to which is a self-splicing intron 48, and then polypeptide-encoding exon 50. Regulatory domain 46 lies within leader region 49. Self-splicing intron 48 thus lies between regulatory domain 46 and exon 50, and is flanked on its 5' side by a ribosome recognition sequence 56, and on its 3' side by an AUG codon 66. An inhibitor region 52 within regulatory domain 46 is complementary to a substrate region 54 at the junction between leader region 49 and self-splicing intron 48. Within the regulatory domain, on the 3'-side of the inhibitor region, is a nucleation region 45 which is contiguous with the inhibitor region 52 and complementary to a region of the signal nucleic acid immediately adjacent to the anti-inhibitor region referred to as the anti-inhibitor extension. The regulatory region may also include an inverted repeat 47 on the 5'-side of the inhibitor region. Substrate region 54 includes ribosome recognition sequence 56, a 5'-splice junction 58, and a stabilizer region 60. Self-splicing intron 48 is overlapped by a 5'-splice junction 58, and a 3'-splice junction 64 adjacent to AUG codon 66, and includes an internal guide sequence 62.

Referring to Fig. 2A, when inhibitor region 52 hybridizes to substrate region 54 a base-paired domain 70 forms preventing 5'-splice junction 58 from interacting

with internal guide sequence 62. The inverted repeat can fold so as to create a stabilizer hairpin 63.

In the presence of a signal nucleic acid, an intermolecular base-paired domain forms between the anti-inhibitor and anti-inhibitor extension regions of the signal nucleic acid and the inhibitor and nucleation regions of the responsive RNA molecule. This interaction frees 5'-splice junction 58 allowing it to interact with internal guide sequence 62. Under these circumstances, a self-splicing reaction occurs. Thus, referring to Fig. 2B, signal nucleic acid 71 having a 3'-end 72 and a 5'-end 73 includes an anti-inhibitor region 74 and an anti-inhibitor extension 77 which hybridize to inhibitor region 52 and nucleation region 45 forming base-paired domain 75.

The self-splicing reaction removes all of the self-splicing intron. The spliced molecule now can produce the encoded polypeptide from exon 50 because the ribosome recognition sequence is now in close juxtaposition to the initiation codon of the polypeptide encoding exon allowing utilization of the initiation sequence as the first codon of a polypeptide.

Referring to Fig. 2C, spliced molecule 90 includes 5'-end 42, 3'-end 44, leader region 49, exon 50, ribosome recognition sequence 56, initiation codon 66, and fused splice junction 95 containing a small portion of 5' splice junction 58 and a small portion of 3' splice junction 64.

Any intron known to have self-splicing activity can be adapted for use as a responsive RNA molecule. Suitable self-splicing RNA can be derived form the nuclear pre-rRNA of <u>Tetrahymena</u>, the mitochondrial pre-rRNA of <u>Saccharomyces</u> and <u>Neurospora</u>, the introns of <u>Argobacterium</u> or <u>Azoarcus</u>, and the mitochondrial pre-mRNA of <u>Saccharomyces</u> or other equivalent group I self-splicing RNAs. Group II introns can also be used in this invention, or any RNA which has at least RNA cleavage activity. RNA ligase activity can be provided by other RNA molecules or their equivalent.

Once a self-splicing RNA has been selected it must be correctly positioned between the ribosome recognition sequence site and the start codon of the polypeptide encoded so that after the self-splicing reaction has occurred the ribosome recognition sequence is positioned correctly relative to the start codon. In eucaryotes translation generally begins at the most 5' AUG of a capped RNA providing that the sequence surrounding the AUG conforms to A/GNNAUGG. Accordingly, the responsive RNA molecule must be designed so that this sequence appears only after splicing has occurred. Moreover, an AUG or other codon in a favorable sequence context can be included in the intron so that it is recognized and used as the 5' most translation initiation site. The inhibitory effect of this upstream AUG on translation initiation at the downstream site will be relieved only upon removal of the intron by self-splicing, thus ensuring that no scanning ribosomal subunits reach the downstream initiation site from which translation of the toxic protein would occur.

In a variation on this type of responsive RNA molecule the self-splicing intron is placed so as to interrupt a polypeptide-coding sequence. As illustrated in Fig. 2D, this molecule has an intron located between two exons that together encode the desired polypeptide. If the intron includes a stop codon, translation will be blocked. Even if the intron does not encode a stop codon, translation of the intron may be out-of-frame with the downstream exon and/or will add amino acids to the polypeptide that will likely destroy activity. Removal of the intron results in the fusion of the two exons and formation of a translatable nucleotide sequence coding for a polypeptide having the desired activity.

Referring to Fig. 2D, responsive RNA molecule 40 has a 5'-end 42 and a 3'-end 44. The polypeptide is encoded in two regions, 50 and 51, separated by self-splicing intron 48. Intron 48 is overlapped by a 5'-splice

junction 58, and a 3'-splice junction 64 and includes internal guide sequence 62. The protein-coding region 50 is preceded by a ribosome recognition sequence 56 and a translational initiation codon 66. An inhibitor region 52 lies within exon 50 and is complementary to substrate region 54 which overlaps the 3'-end of region 50 and the 5'-splice junction 58 and includes stabilizer region 60. Flanking the inhibitor region on its 5' side is nucleation region 45 that is contiguous with the inhibitor region and is complementary to regions in the signal nucleic acid immediately adjacent to the anti-inhibitor region.

Referring to Fig. 2E, when the inhibitor region 52 hybridizes to substrate region 54 a base-paired domain 70 forms and thus prevents the 5'-splice junction 58 from interacting with the internal guide sequence 62.

Referring to Fig. 2F, signal nucleic acid 71 having a 3'-end 72 and a 5'-end 73 and including an anti-inhibitor region 74 and an anti-inhibitor extension 77 hybridizes to the inhibitor region 52 and nucleation region 45. The intermolecular base-paired domain 75 is formed. Under these circumstances, the 5'-splice junction 58 is free to interact with the internal guide sequence 62 and self-splicing occurs.

Referring to Fig. 2G, the self-splicing reaction removes all of the self-splicing intron 48 leaving the fused spliced junction 95 which contains portions of the 5'-splice junction 58 and the 3'-splice junction 64.

Other strategies, for example, where the substrate and/or inhibitor regions are contained within the intron, may be used so that upon splicing these elements are completely removed. When the substrate or inhibitor domains remain in the protein-coding regions, their sequences must be carefully chosen to preserve the biological activity of the encoded protein. The degeneracy of the genetic code, the possibility of guanosine-uridine base-pairs and conservative amino acid changes that do not eliminate the protein's activity will

all be considered. Moreover, it is known that many proteins contain regions not essential to their inherent activity and that amino acid changes and/or additions in these areas do not result in a drastic loss of biological activity. The placement of the substrate and/or inhibitor domains in such a region simplifies the choice of the anti-inhibitor containing signal RNA since changes to the protein-coding sequence might be more easily tolerated.

The requirement that the inhibitor region be complementary to both the anti-inhibitor region and the substrate region places certain constraints on the sequences of these regions. First, as noted above, the substrate region does not have to have the same sequence as the anti-inhibitor region of the signal nucleic acid. Since the anti-inhibitor region can be selected but not altered, the anti-inhibitor region must include a sequence identical to the sequence of the 5'-splice junction. The minimal 5'-splice junction in a Tetrahymena rRNA intron is only four nucleotides long. Since any four nucleotide sequence should occur with a probability of 1/64, many potential anti-inhibitor regions will include the sequence of the 5'-splice junction. It is very likely that many different four-base sequences can serve as a 5'-splice junction provided that the sequence of the internal guide region is adjusted to accommodate the changes in the 5'-splice junction (Zaug et al., Nature 324:430, 1986). While it is suitable for the minimal 5'-splice junction to be able to base pair with the internal guide sequence, a complex with a single mis-match can be functional (Zaug et al, Biochemistry 27:8924, 1988).

The base-paired domain formed by hybridization of the inhibitor region and the substrate region must be more stable than the base-pairing that occurs between the 5'-splice junction and the internal guide sequence during a splicing reaction. This can be accomplished by choosing an inhibitor region and substrate region that will hybridize to form a base-paired domain longer than that

formed by hybridization of 5'-splice junction to the internal guide sequence. The substrate region is designed to include a stabilizer region that extends the homology between the substrate region and the inhibitor region sequence beyond the 5'-splice junction. This stabilizer region can be located just 3' of the 5'-splice junction in the case of self-splicing introns located between the ribosome recognition sequence and the initiation codon. This arrangement insures that the stabilizer domain will be removed as part of the splicing reaction and will not interfere with the relationship between the ribosome recognition sequence and the initiation codon. The ribosome recognition sequence can also be included within the region which base-pairs with the inhibitor region, but there is no requirement that this be the case. In the case of a self-splicing intron which is inserted between exons which encode portions of the same polypeptide, the stabilizer region should preferably be located within the intron, i.e., on the 3'-side of the 5'-splice junction so that it will be removed along with the rest of the intron.

It is important that the inhibitor/substrate base-paired domain be disrupted only by the signal nucleic acid and not by other nucleic acids present in cell. As discussed above, for the first type of responsive RNA molecule, this means that the intramolecular base-pair formation must be extensive enough to be disrupted only by a unique nucleic acid. This requirement can make it difficult for the signal nucleic acid to disrupt the intramolecular duplex. As outlined above, the inclusion of a nucleation region adjacent to the inhibitor region will favor hybridization of the inhibitor region to the anti-inhibitor region.

Many arrangements of the regulatory domain of the self-splicing intron and the exon will be useful. As noted above, the self-splicing intron can be located between two exons; under these circumstances while the most 5' exon of the unspliced molecule can be translated

a complete functional polypeptide cannot be produced. The inhibitor region can be located on the 5'- or the 3'- side of the self-splicing intron or possibly within the intron itself. Since RNA is synthesized in the 5' to 3' direction, it is preferred to locate the inhibitor region on the 5'-side so that the inhibitor will be synthesized and have an opportunity to hybridize to the 5'-splice junction before the production of the internal guide sequence. The inhibitor could be located on the 3'-side of the self-splicing intron if folding of the RNA to form the splicing complex is slow compared to rate of synthesis of the inhibitor region.

It is preferred that the self-splicing reaction be specific and accurate; if the splice occurs at the wrong location, the ribosome binding site will be positioned incorrectly. In the case of a self-splicing intron located between two exons, incorrect splicing may result in an out-of-frame fusion of the polypeptide encoding sequences. Self-splicing introns in which the distance between the internal guide sequence and the 5'-splice junction is relatively short tend to catalyze more accurate splicing reactions. It is also important to insure that there are no sequences that will be recognized as alternative 5'-splice junctions.

The above described responsive RNA molecules can be prepared by any standard methodology. For example, the RNA can be produced by a transcription of a DNA molecule, either <u>in vivo</u> or <u>in vitro</u>. Generally, the RNA molecule will be produced by construction of a plasmid or viral DNA which includes sequences encoding the responsive molecule, appropriate sequences for regulated transcription of the responsive RNA molecule, and appropriate sequences for replication of the DNA. In constructing the RNA molecule, the general considerations are described above. From a practical viewpoint, it is generally preferred to identify an appropriate RNA molecule having enzymatic activity which is able to cleave itself or other RNA molecules and

is preferably able to splice those two RNA molecules together, e.g., a self-splicing RNA molecule. The DNA encoding this RNA molecule is then modified to change the encoded 5'-splice junction and the internal guide sequence as required within the limitations described above so that the encoded 5'-splice junction is complementary to part of the inhibitor region of the responsive RNA molecule. The transcribed RNA molecule is then caused to be ligated to RNA which encodes the desired polypeptide and to RNA which includes an appropriate regulatory domain. If required, nucleation sites and inverted repeats can be designed into the regulatory domain.

The experiments discussed in the following Examples 3-7 describe preparation of responsive RNA molecules containing inactive introns which can be reactivated by the presence of specific signal RNAs. The responsive RNA molecules were prepared from the self-splicing intron or intervening sequence (IVS) in the rRNA of Tetrahymena thermophila. For the IVS to self-splice requires the proper folding of the core structure of the IVS RNA. Included in this required conformation is a base-paired region known as P(1) that encompasses the 5'-splice site (Fig. 3). In P(1), the internal guide sequence in the IVS base pairs with the adjacent portion of the 5' exon to form a stable stem-loop structure. The 5'-splice site is located within this stem. The ability of the IVS RNA to self-splice relies on the ability of the P(1) stem to form.

A natural sequence just upstream of the 5'-splice site can also form a hairpin structure with the exon sequence immediately adjacent to the 5'-splice site (Fig. 3). The stem-loop required for self-splicing, P(1), and this alternative stem-loop, termed P(-1), are mutually exclusive since the 5' exon sequence immediately adjacent to the splice site is included in both structures. The alternative stem-loop structure, P(-1), can be made more stable by extending its stem region. See Woodson and

Cech, Biochemistry, 30:2042, 1991, reporting results of a one-nucleotide change in the 5' exon (A to C change at position -14 relative to the 5'-splice site). In that mutant, self-splicing was reported to be decreased. Conversely, RNAs containing mutations in the 5' exon which either diminished the relative strength of P(-1) or abolished it completely reportedly showed an increase in self-splicing activity. Three mutants which contain sequence changes in the 5' exon, which were predicted to strengthen the alternative structure, P(-1), were made. In all three mutants, the level of *in vitro* self-splicing (as judged by the formation of ligated exons) was decreased relative to a parent construct in which the natural 5' exon sequence is present. One mutant, in which the stem of P(-1) has been lengthened by 5 additional base-pairs, exhibits no detectable self-splicing activity *in vitro*.

Applicant demonstrated that self-splicing activity can be recovered even in this strong, non-splicing mutant by the addition of signal RNAs complementary to the upstream 5' exon sequence (inhibitor region) involved in the alternative structure. By binding to the 5' portion of the P(-1) stem, these signal RNAs disrupted P(-1) and left the sequence immediately adjacent to the 5'-splice site in single-stranded form, fully capable of hybridizing to the internal guide sequence in an active, self-splicing conformation containing P(1).

Example 3: Plasmid Construction and DNA preparation

The source of the IVS-containing fragment used to prepare the responsive RNA molecules was plasmid pTT1A3T7 (obtained from Dr. A. Zaug; equivalent such plasmids are readily constructed and this plasmid is used only for purposes of illustration of the invention), which contains the 482-bp *Tha*I fragment of *Tetrahymena thermophila* rDNA inserted into the *Hin*dIII site of pT7-2 (U.S. Biochemical Corporation, Cleveland, Ohio) on *Hin*dIII linkers. This

fragment contains rDNA sequence corresponding to 32 nt of 5' exon, the 413 nt IVS, and 37 nt of 3'-exon. The HindIII fragment of pTT1A3T7 was isolated and inserted into the HindIII site of pTZ19R (United States Biochemical Corporation, Cleveland, OH) to generate a plasmid containing the IVS and a small portion of the natural rDNA sequence inserted into the first few codons of the lacZ' gene, the α-complementation fragment of the β-galactosidase gene. It has been reported previously by others (Been and Cech, Cell 47:207, 1986; Price and Cech, Science 228:719, 1985; Waring et al., Cell 40:371, 1985), that β-galactosidase activity in *E. coli* relies on the ability of the IVS RNA to excise itself and ligate the lacZ' coding region in frame so as to produce a translatable mRNA product. *In vitro* mutagenesis was carried out on the pTZ19R derivative containing the rDNA insert to generate a clone in which the corresponding lacZ' RNA would self-splice and maintain the correct reading frame. In addition, a potentially useful SalI site was created in the 3'-exon and an in-frame AUG in the 3'-exon was destroyed to insure that it not be used as a translation start site. The final DNA sequence and correct reading frame of the 3'-exon from the 3'-splice site (▲) to the HindIII site (underlined) in the vector sequence is shown below.

pTETBLU

▲ T AAG GTA GCC AGC CGT CGA CAT CTA ATT AGT GAC GCA AGC TT

pTETBLU DNA was then used as the parent for a series of splicing mutants in which changes were made by *in vitro* mutagenesis in the 5' exon sequence to improve the base-pairing ability in the alternative P(-1) stem-loop structure. Care was taken to maintain the correct reading frame in the spliced RNA product and to avoid the creation of translational start or stop codons. The resulting sequence changes made in the 5' exon RNA and the RNA

alternative structures predicted to form are shown in Fig. 3.

All site-specific mutations were generated using the *in vitro* Mutagenesis Kit from United States Biochemical Corporation. DNA oligonucleotides were made on an Applied Biosystems 394 DNA/RNA Synthesizer using phosphoramidite chemistry and purified using OLIGOCLEAN™ columns (United States Biochemical Corporation) prior to use as mutagenic oligonucleotide. Plasmids were maintained in strain MV1190 (*E. coli ▲ (srl-recA) 306::TN10 ▲ (lac-pro) thi-supE (F' pro A+B+ lacI*^{Q} *lacZ ▲ Mi5 traD36*). Each plasmid was verified by DNA sequencing (Tabor and Richardson, *Proc. Natl. Acad. Sci. USA* *84*:4767, 1987).

Plasmids for use as *in vitro* transcription templates were purified by Qiagen (Qiagen Inc., Chatsworth, CA) maxi-column preparation as described by the manufacturer except that the final DNA preparation (400 μl) was extracted two times with an equal volume of phenol, once with chloroform, and ethanol precipitated in the presence of 0.25 M Tris-HCl, pH 7.5. The plasmids were linearized by cleavage with either *Eco*RI or *Bam*HI to generate templates on which runoff T7 transcription will yield full-length RNA of 548 or 527 nt, respectively. (The T7 promoter sequence is located immediately upstream of the polycloning site and within the coding sequence of β-galactosidase.)

Example 4: Signal RNAs

Short signal RNAs (11-26 nt) were chemically synthesized on an Applied Biosystems 380B DNA synthesizer using phosphoramidite chemistry. Prior to use, the signal RNAs were desalted using a C_{18} SEP-PAC® cartridge (Millipore Corporation), gel-purified and quantified by absorbance at 260 nm. Signal RNAs were stored at -20°C in 1 mM EDTA, 10 mM Tris-HCl (pH 7.5). The sequences of the signal RNAs specific for precursor RNA from PTET1419 and pTET21-24 (see FIG. 3) are given below:

pTET1419	4S	3'	GCCGCUCUCAG	5'
	4S3	3'	GCCGCUCUCAGUGAU	5'
pTET21-24	8S4	3'	CGCCCAUUUAAAUCUCUCAGUGAUA	5'
	12S	3'	CGGAAACGCCCAUUUAAAUCUCUCAG	5'

These signal RNAs are complementary to the upstream exon sequence which forms the 5' side of the P(-1) stem in the given construct. The underlined nucleotides correspond to the portion of the signal sequence that will base pair with 5' exon sequence involved in the P(-1) stem, the remaining nucleotides base pair either with nucleotides at the base of the stem or in the loop. For example, signal RNA 4S3 will base pair with 4 nt 5' to the base of the stem in pTET1419 RNA, all the nucleotides included in the 5' side of the P(-1) stem and 3 nucleotides in the loop.

In pTET14 RNA (see FIG. 3), a U to C change at -14 relative to the 5'-splice site allows the formation of an extra C-G base-pair to lengthen the P(-1) stem. This particular sequence change was reported by Woodson and Cech (Woodson and Cech, Biochemistry 30:2042, 1991) to decrease self-splicing activity of a short precursor RNA. pTET1419 RNA has an additional nucleotide change (G to A at -19) which allows P(-1) to form a more stable stem by creating an A-U base pair in place of a less stable G-U base pair. Finally, pTET21-24 RNA has a very stable P(-1) stem generated by 4 additional nucleotide changes (at positions -21 to -24 relative to the splice site). Calculated free energies at 37°C for these structures, based on the most current values in the literature (Freier et al., Proc. Natl. Acad. Sci. USA 83:9373, 1986; Jaeger et al., Proc. Natl. Acad. Sci. USA 86:7706, 1989), are also given in Fig. 3. In all of these constructs, nucleotide changes were made in the upstream 5' exon only, without altering the IVS or the 13 nt at the 3' end of the 5' exon.

On templates linearized with EcoRI or BamHI, full-length transcription from the T7 promoter yielded

transcripts of 548 and 527 nt, respectively. These differed only in the length of their 3'-exon (92 vs. 71 nt), but had equivalent length 5' exons (43 nt) and IVS RNA (413 nt). Correct ligation of the 3'-exon to the 5' exon with excision of the IVS yielded an RNA of 135 nt for the <u>Eco</u>RI runoff transcript and 114 nt for the corresponding <u>Bam</u>HI transcript. The appearance of ligated exons is an indication of the level of self-splicing supported by a particular IVS-containing construct.

<u>Example 5: Decreasing Self-Splicing by Increasing Stability of P(-1).</u>

<u>In vitro</u> transcription was performed as follows. Transcription reactions using T7 RNA polymerase were carried out in transcription buffer (40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine) containing 500 μM each NTP and ~10 μCi [α^{32}P]CTP. Individual reactions (10 μl total volume) contained 0.1 μg linearized plasmid template and 20-30 U T7 RNA polymerase. After 30 minutes at 30°C, 2 μl of each sample was removed and mixed with 2 μl buffered formamide containing xylene cyanol FF and bromphenol blue (formamide/dye mix). The remainder of the sample was warmed to 37°C, and 2 μl of 1 M NaCl, 20 mM $MgCl_2$, 1 mM GTP was added to adjust the reaction conditions to better support splicing. After 15 or 60 minutes as noted, 2.5 μl samples were removed and mixed with 2.5 μl of the formamide/dye mix. Samples were analyzed on denaturing gels containing 4% (19:1) acrylamide:bisacrylamide and 7M urea in 0.4 X TBE (TBE is 89 mM Tris, 89 mM boric acid, 0.025 mM EDTA). Electrophoresis was carried out at 30-60 watts using 0.4 X TBE as running buffer. Gels were exposed to Kodak XOMAT XAR-5 film.

For gel purification of ^{32}P-labelled, precursor RNAs, transcription reactions were scaled up 2.5- to 10-fold and incubated 1-2 hours at 37°C. In some cases, the concentration of each NTP was increased to 2.5-3 mM in an

attempt to reduce self-splicing during the transcription reaction and thereby maximize the recovery of full-length transcripts. An equal volume of formamide/dyes was added to the completed reaction and the entire reaction was loaded onto a denaturing gel as described above. After visualization by autoradiography, the region of the gel containing the full-length transcript was excised and placed in 0.5-1 ml 0.5 M ammonium acetate, 1 mM EDTA. After 12-16 hours at 4°C, the eluent was removed and the RNA precipitated by the addition of 2.5 volumes of ethanol. The final RNA pellet was resuspended in 1 mM EDTA, 10 mM Tris-HCl (pH 7.5) and stored at -20°C.

Transcription using the parent plasmid and the modified constructs as templates was carried out in the presence of $[\alpha^{32}P]$CTP to generate ^{32}P-labelled transcripts that could be analyzed for their ability to self-splice (Fig. 4, 0 min). Full-length transcripts (FL), a slight amount of IVS RNA (IVS), and additional "intermediate" RNA products (*), were present for all templates. A small amount of an RNA product of the appropriate length to be ligated exons (LE) from the EcoRI run-off transcript (135 nt) as well as from the BamHI run-off transcript (114 nt) was also visible, and indicated that a limited amount of splicing could occur under these transcription conditions. This faint band decreased in intensity with the order pTETBLU>pTET14>pTET1419 and was not visible in pTET21-24.

From analysis of the resultant RNA products, it is clear that transcription of the parent plasmid, pTETBLU, generated transcripts capable of efficient self-splicing. This is evidenced by an increased amount of ligated exons 15 and 60 minutes after adjusting the conditions to better support splicing.

By comparison of the amount of ligated exon produced, it is apparent that transcripts from pTET14 and pTET1419 were still capable of self-splicing, although less efficiently than transcripts from the parent pTETBLU. Both pTET14 and pTET1419 produced fewer ligated exons than

pTETBLU when shifted to splicing conditions, and of these two mutants, pTET1419 was the least efficient. Under the same conditions, however, transcripts from pTET21-24 did not appear to self-splice. No ligated exons were visible for pTET21-24 precursors after conditions were altered to support splicing. The relative observed ability of these three mutant constructs to self-splice, then, follows the order expected based on the increasing stability of the P(-1) stem, i.e., there is a negative correlation between the strength of the P(-1) stem and the RNA's ability to self-splice. Moreover, the presence of the highly stabilized P(-1) stem in pTET21-24 reduced in vitro splicing to undetectable levels.

Under splicing conditions, a number of RNA products in addition to the ligated exons were visualized. As expected, splicing of the pTETBLU transcript generated a significant amount of the excised IVS RNA in its various forms (circular and linear IVS and the shortened forms lacking the 5' 15 or 19 nt). Some of these products were visible for the mutant transcripts as well, even for pTET21-24 where no ligated exons were visible. The presence of these IVS products may reflect the ability of these mutant RNAs, which are to various degrees misfolded at the 5'-splice site due to a stronger than normal P(-1) stem, to still support hydrolysis at their 3'-splice site (See Woodson and Cech, Biochemistry 30:2042, 1991). Although no released 3'-exon was visible, one RNA product that was greatly enhanced in the mutant RNA lanes (indicated with an asterisk in Fig. 4), was of the appropriate size to represent the 5' exon-IVS RNA. This 5' exon-IVS RNA would still be expected to undergo circularization reactions, producing the linear IVS products (L-15 and L-19) seen on the gel. The short RNA indicated with an arrowhead is unidentified. This RNA increased in intensity after the switch to splicing conditions. It also seemed to increase in abundance as

the ability of the precursor RNA to self-splice decreased, and thus was most prominent in the pTET21-24 RNA lanes.

It is clear from the lack of ligated exons in the pTET21-24 lanes that this mutant was unable to undergo correct ligation of the two exon products. The apparent side reactions of the mutant IVS-containing RNAs (e.g., the formation of the RNA product labeled with the asterisk) when unable to undergo a correct splicing reaction may be able to be used advantageously. For example, this "self-destruction" may be beneficial for IVS-containing mRNAs that encode toxins where rapid turnover of the message would further diminish the possibility that a toxin be produced in the absence of the proper signal.

Example 6: Reactivity of Splicing Reaction by Signal RNA

Gel-purified, full-length RNA precursors were subjected to splicing conditions in the absence or presence of signal RNAs to test the ability of short RNAs complementary to the upstream 5' exon sequence to disrupt the P(-1) structure and thereby allow the active P(1) structure to form.

Splicing reactions using gel-purified precursor RNAs were carried out by incubating 0.1-0.25 pmole of ^{32}P-labelled transcription 10 μl splicing buffer (200 mM NaCl, 200 μM GTP, 30 mM Tris-HCl, pH 7.5) in the presence of 0 to 1000-fold molar excess of signal RNAs. After warming to 37°C, $MgCl_2$ was added to 5 mM to initiate the splicing reaction. Incubation periods ranged from 10 to 120 minutes at 37°C, at which times samples were removed and mixed with an equal volume of formamide/dye. Samples were analyzed on denaturing gels as described above.

If self-splicing were reactivated, more ligated exon products would be expected to be produced in the presence of these signal RNAs than in their absence. Results of experiments demonstrating reactivation of the splicing

reaction are given for pTET1419 RNA in Fig. 5 and for pTET21-24 RNA in Fig. 6.

As seen previously in Fig. 5, incubation of pTET1419 RNA under splicing conditions in the absence of any signal RNA generated a small amount of ligated exon product. With gel-purified transcript, this was again the case (Fig. 5). It may be that the P(-1) stem in pTET1419 RNA is not stable enough to completely inhibit the formation of P(1), so a small amount of splicing still occurred. The amount of ligated exons produced increased, however when either of two specific signal RNAs was present in incubation. Even with an extremely low signal-to-transcript ratio (0.1:1), a slight elevation in the amount of ligated exons was seen. As the signal-to-transcript ratio was increased (up to 1000:1), the production of ligated exons also increased. These experiments showed that the ability of pTET1419 RNA to correctly self-splice and produce ligated exons responds directly to the presence of a specific signal RNA, and that a significant level of self-splicing is recovered.

A similar response to signal RNAs was seen with gel-purified pTET21-24 RNA (Fig. 6). As noted before, with pTET21-24 RNA, no ligated exons were visible when the transcript was incubated alone (see also Fig. 4). This indicates that the P(-1) stem in pTET21-24 RNA is sufficiently stable to completely inhibit the formation of P(1). Upon addition of either of two signal RNAs (8S4 or 12S) specific for this transcript, however, ligated exons are produced. That the ^{32}P-labelled RNA products are ligated exons can be seen by comparing their length to that of ligated exons produced from pTETBLU RNA. Splicing of transcripts produced from <u>Eco</u>RI-digested templates produced ligated exons of 135 nt in length. Transcripts from templates linearized with <u>Bam</u>HI produced ligated exons that were correspondingly shorter (114 nt). Thus, even though the splicing reaction was turned completely "off" in the pTET21-24 RNA itself, it was still possible

to reactive the splicing reaction with a specific signal RNA.

For the EcoRI runoff transcripts shown on the left of Fig. 6, there was a second major product (indicated with a dot) that also seemed to respond to the presence of the signal RNAs. This RNA is shorter than the correctly ligated exons, and at this time its origin in unknown. Splicing at an alternative site or a specific breakdown of the RNA are possibilities.

Example 7: Colony Color Assay

When grown on LB or B agar plates containing 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal), a chromogenic substrate of β-galactosidase, pTETBLU-containing colonies are dark blue as expected for a colony producing β-galactosidase. Since the coding region of the α-complementation fragment of β-galactosidase on pTETBLU is interrupted by the Tetrahymena IVS, this RNA must be correctly self-splicing in order to produce an active α-fragment. If self-splicing is not occurring, stop codons present in all three reading frames in the IVS would not allow translation into the downstream portion of the gene. For comparison, a control plasmid (pTETULB) in which the intron-containing HindIII fragment from pTETBLU is inserted into pTZ19R in the reverse orientation was constructed. For this control, where no splicing can occur due to the wrong orientation, the resulting colonies are white.

Theoretically, then, cells containing mutants which are deficient in splicing should produce lighter blue colonies, while colonies of non-splicing mutants would be white. Under standard growth conditions, cells containing pTET1419 and pTET21-24 mutants grew as colonies that were considerably lighter in color than cells containing the parent plasmid pTETBLU, but not white. This appears to indicate that even the strongest non-splicing mutant, pTET21-24 (as judged by its inability to form ligated

exons *in vitro*) is still capable of forming the minimal amount of spliced message necessary to support translation of a level of an α-fragment of β-galactosidase that could confer blue color to the colonies. Other scientists have noted β-galactosidase activity (blue colony color) with IVS-containing constructs in which self-splicing should have left the β-galactosidase message in an untranslatable frame (Been and Cech, Cell 47:207, 1986; Price and Cech, Science 228:719, 1985). It may be that alternative splice sites exist.

For a more quantitative determination, β-galactosidase assays were carried out on plasmid-containing cells growing in culture. (Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972). For this assay, o-nitrophenyl-β-D-galactoside (ONPG) was used as the chromogenic substrate because its product after cleavage with β-galactosidase can be measured spectrophotometrically. A control plasmid (pTETULB) was constructed in which the intron-containing *Hin*dIII fragment from pTETBLU was inserted into pTZ19R in the reverse orientation and was used to determine background levels of spontaneous breakdown of ONPG. In these experiments, cells containing either the parent plasmid or the splicing mutants were grown under inducing conditions (*i.e.*, in the presence of IPTG, a lactose analog). Production of active β-galactosidase in cells containing the pTET1419 and pTET21-24 splicing mutants was reduced to a few percent of the parental values, thus indicating that the changes in the RNA were reflected, not only by a decrease in the amount of *in vitro* self-splicing, but by a concomitant decrease in the amount of active protein produced in the *E. coli* cell.

Use

The responsive RNA molecules of the invention are useful for producing plant cells that respond to the presence of a given virus. In many instances there is no way to prevent viral infection of such cells. The

molecules of the invention will allow creation of plant lines that are resistant to any given virus in that any plant cells which become infected will be destroyed before the virus is able to spread to other cells.

This section describes the methods by which a responsive RNA can be used to affect the physiological state or viability of a particular cell type. In the case of responsive RNA molecules that are regulated by the formation of a base-paired domain within a protein-coding region the method requires construction of a responsive RNA which encodes a protein which will affect the physiology or viability of a cell; and identification of an signal RNA which is specific to the cell type, i.e., an RNA molecule which carries a nucleotide sequence that is only present or accessible in the RNA population of the cell type which is to be affected. For responsive RNA molecules regulated by self-splicing introns the method requires construction of a responsive RNA which encodes a protein which will affect the physiology or viability of a cell. The active protein must be translated from the spliced message and not the unspliced message. It also requires identification of a signal RNA which is specific to the cell type, i.e., an RNA molecule which carries a nucleotide sequence that is only present or accessible in the RNA population of the cell type which is to be affected.

For example, a responsive RNA can be designed to specifically kill: virus-infected plant cells containing viral RNA and not uninfected cells; cells containing mutant RNA and not cells containing wild type RNA; cells in a particular tissue and not other kinds of cell in the plant.

The efficacy of such a responsive RNA in altering the physiological state of a cell will depend upon the responsive RNA being delivered to the location in the cell where the signal nucleic acid resides; the responsive RNA having all of the nucleoside sequences required for all

the processes leading to production of the encoded protein including splicing, poly-A addition, capping, transport across the nuclear membrane, and translation initiation; and the responsive RNA may also carry sequence elements which confer stability to RNA in the nucleus as well as the cytoplasm.

A responsive RNA molecule can be delivered into a cell in the form of RNA or in the form of a gene made of DNA or RNA. Delivery of RNA into a cell can be accomplished by needle injection, electroporation, polyethyleneglycol precipitation, or by the use of liposomes including those made of cationic lipids. Delivery of the responsive RNA in the form of a gene can be accomplished by the use of a nonvirulent virus or bacterium. This would require the insertion of the responsive RNA-encoding gene along with the transcriptional or replicative signal elements into the genome of the virus. Retroviruses, polyoma viruses, and vaccinia virus have been engineered which are capable of delivering and expressing genes, and other viruses could be developed and used for this purpose.

Another general method of using a responsive RNA to control the physiology of an organism or a particular cell type involves a responsive RNA gene integrated into the cellular genome via any plant transformation technique, e.g., Agrobacterium tumifaciens. The activation of splicing of the responsive RNA could be caused by exogenously added polynucleotides.

Other embodiments are within the following claims.

Claims

1. A responsive RNA molecule having a ribosome recognition sequence, a regulatory domain, a substrate region, and encoding, in one or more protein-coding regions, a polypeptide; said regulatory domain comprising an inhibitor region and complementary to said substrate region; said inhibitor and substrate regions being capable of forming a base-paired domain in the absence of a signal nucleic acid; said base-paired domain reducing the level of translation compared to that level observed in the absence of said base-paired domain; said signal nucleic acid having an anti-inhibitory region complementary to said inhibitor region which, when base-paired with said inhibitor region, increases the level of translation of said responsive RNA compared to the level of translation of said responsive RNA observed in the absence of said signal nucleic acid; wherein said signal nucleic acid comprises part of the nucleic acid of a plant cell infecting organism.

2. The responsive RNA of claim 1 wherein said protein-coding region is an exon.

3. The responsive RNA of claim 1 wherein said substrate region comprises part of one said protein-coding region.

4. The responsive RNA of claim 2 wherein said substrate region comprises part of an intron.

5. The responsive RNA of claim 2 wherein said substrate region comprises part of an intron adjacent to the 5'-end of one said exon.

6. The responsive RNA of claim 1 wherein said substrate region includes part of said ribosome recognition sequence.

7. The responsive RNA of claim 6 wherein said ribosome recognition sequence is a ribosome binding site.

8. The responsive RNA of claim 1 wherein said responsive RNA is purified.

9. The responsive RNA of claim 1 wherein said polypeptide modifies cell viability, cell proliferation, transcription of DNA, translation of RNA, or replication of DNA.

10. The responsive RNA of claim 9 wherein said polypeptide has cytotoxic activity or ribonuclease activity.

11. The responsive RNA of claim 10 wherein said polypeptide is selected from the group consisting of the active subunit of diphtheria toxin, the active subunit of cholera toxin, ricin, and the hok, gef, RelF or flm gene products of E. coli.

12. The responsive RNA of claim 14 wherein said intron prevents the complete translation of said one or more exons.

13. The responsive RNA of claim 4 wherein said intron reduces the level of translation of said one or more exons compared to the level of translation of said exon in the absence of said intron.

14. The responsive RNA of claim 4 wherein said intron is located between said ribosome recognition sequence and a protein-coding region.

15. The responsive RNA of claim 4 wherein said first intron is located between two said exons.

16. The responsive RNA of claim 4 wherein said intron is bordered at its 5'-end by a 5'-splice junction and at its 3'-end by a 3'-splice junction.

17. The responsive RNA of claim 15 wherein said substrate region comprises a 5'-splice junction bordering said intron.

18. The responsive RNA of claim 16 wherein said intron catalyzes two RNA cleavage reactions, one within said 5'-splice junction and one within said 3'-splice junction.

19. The responsive RNA of claim 18 wherein said substrate region comprises the 5'-splice junction of said intron.

20. The responsive RNA of claim 19 wherein said inhibitor region reduces the level of occurrence of said cleavage reaction within said 5'-splice junction.

21. The responsive RNA of claim 1 wherein said signal nucleic acid is single-stranded.

22. The responsive RNA of claim 10 wherein said signal nucleic acid is a viral RNA.

23. A DNA molecule encoding the responsive RNA of claim 1.

24. The responsive RNA of claim 19 wherein said responsive RNA comprises a 5'-splice junction RNA of Tetrahymena thermophila having at least one base modified compared to a native 5'-splice junction.

25. A method for specifically interfering with the growth of a plant cell harboring a signal nucleic acid by

introducing into the cell the responsive RNA wherein said responsive RNA comprises a ribosome recognition sequence, a regulatory domain, a substrate region, and encoding, in one or more exons, a polypeptide; said regulatory domain comprising an inhibitor region complementary to said substrate region; said inhibitor and substrate regions being capable of forming a base-paired domain in the absence of a signal nucleic acid; said base-paired domain reducing the level of translation of said responsive RNA molecule compared to the level of translation in the absence of said base-paired domain; said signal nucleic acid having an anti-inhibitor region complementary to said inhibitor region which, when base-paired with said inhibitor region, increases the level of translation of said responsive RNA compared to the level of translation of said responsive RNA observed in the absence of said signal nucleic acid wherein said signal nucleic acid comprises part of the nucleic acid of a plant cell infecting organism.

26. The responsive RNA of claim 1 expressed in a transgenic plant.

27. A plant comprising the responding RNA of claim 1.

FIG. 1.

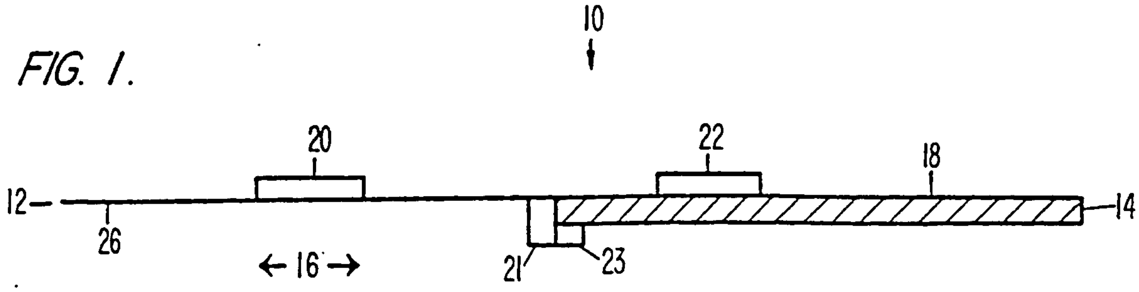


FIG. 1a.

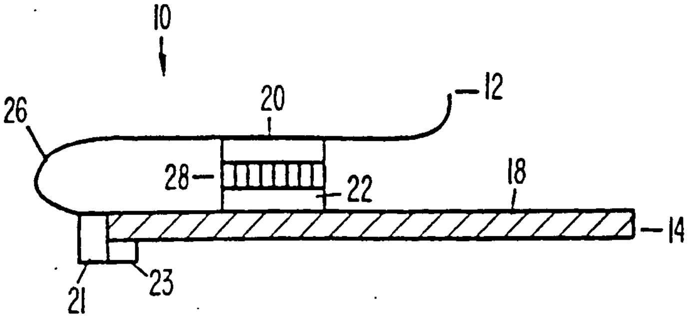


FIG. 1b.

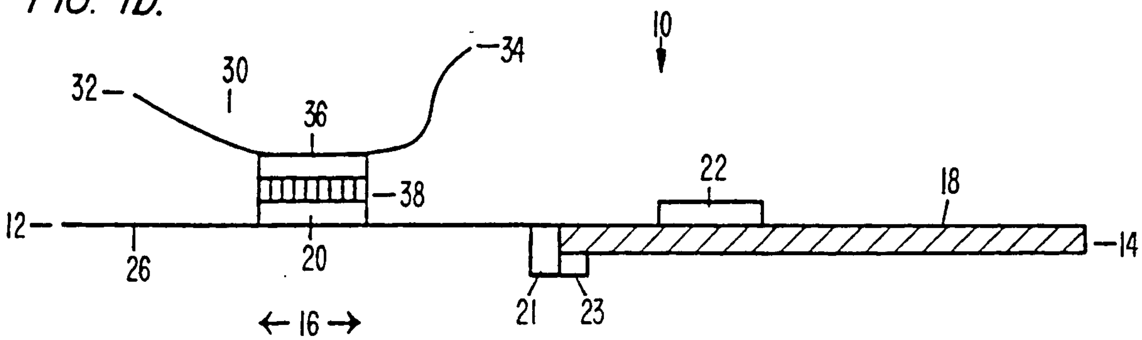


FIG. 1c.

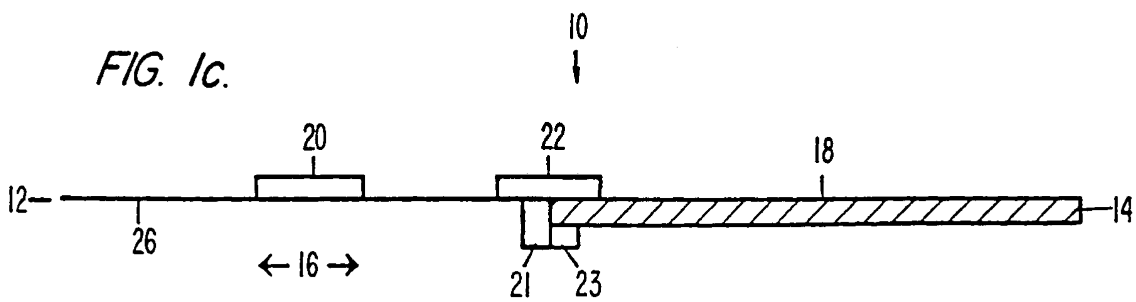


FIG. 1d.

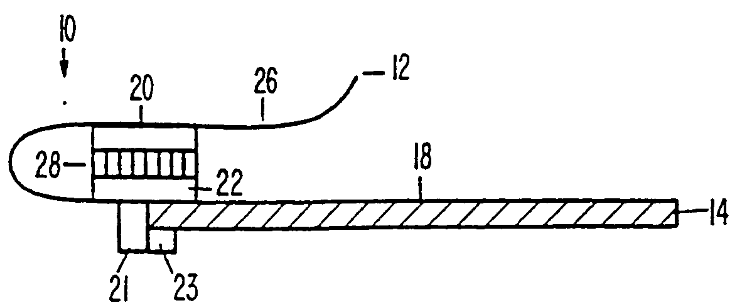


FIG. 1e.

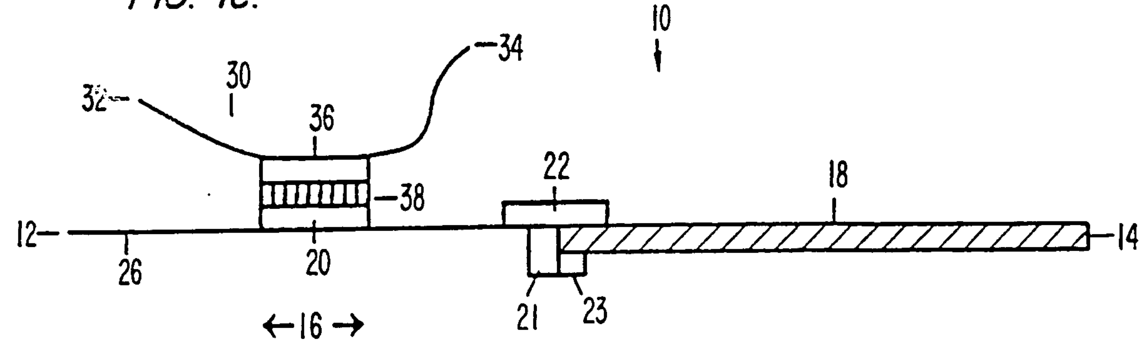


FIG. 1f.

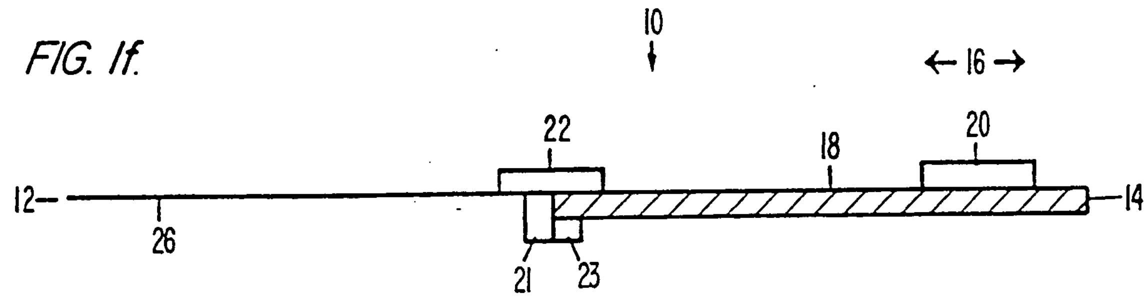


FIG. 1g.

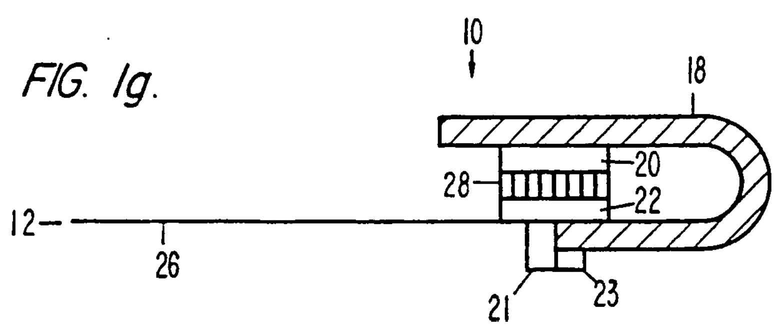
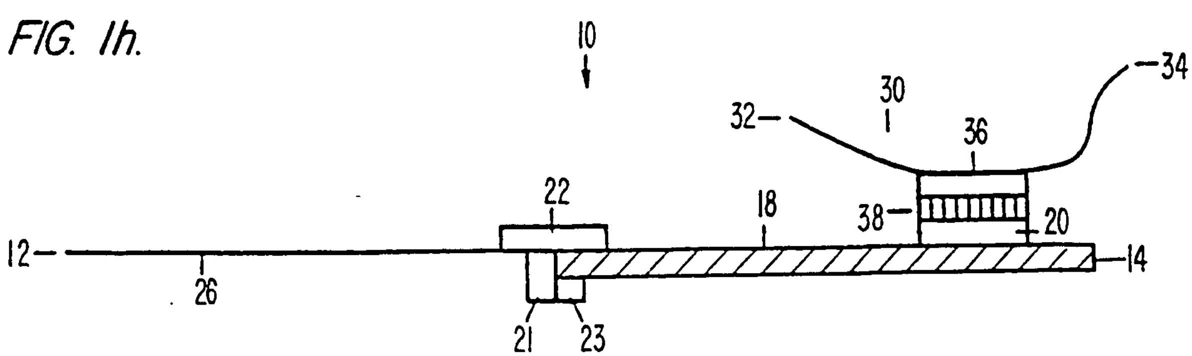


FIG. 1h.



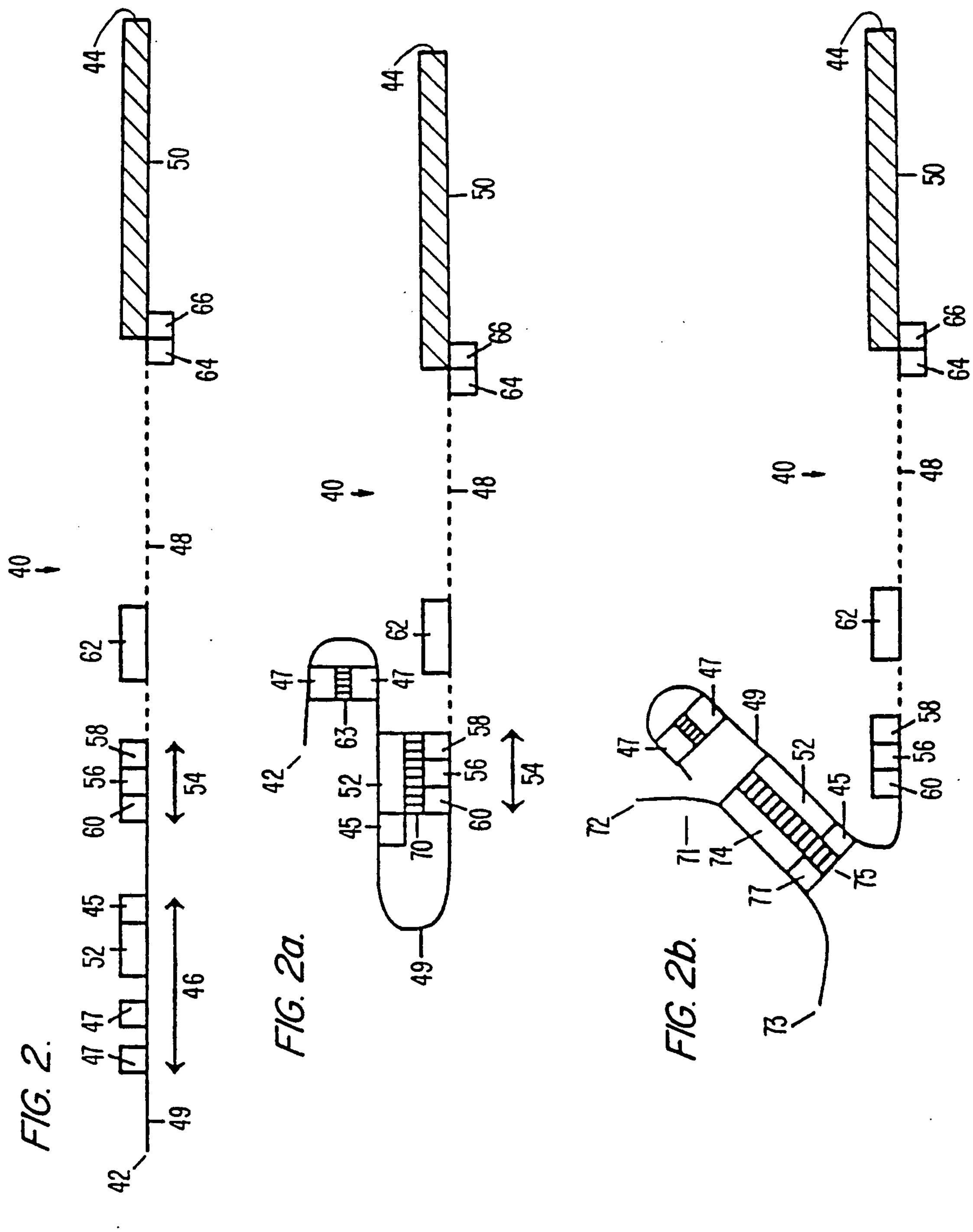


FIG. 2.

FIG. 2a.

FIG. 2b.

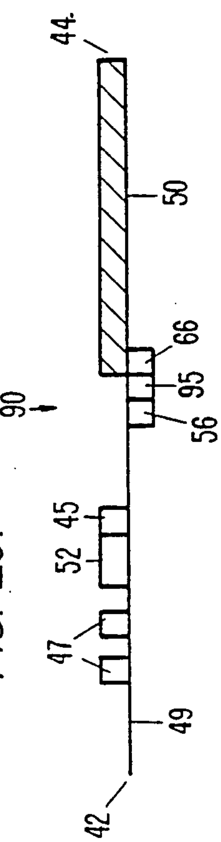

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FIG. 2c.

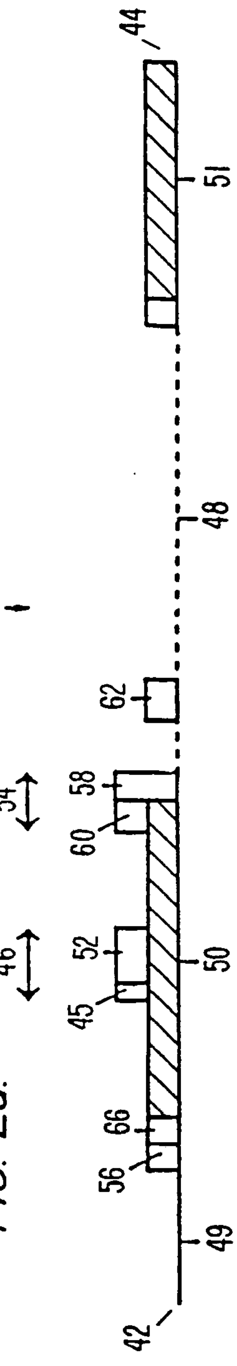
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FIG. 2d.

FIG. 2e.

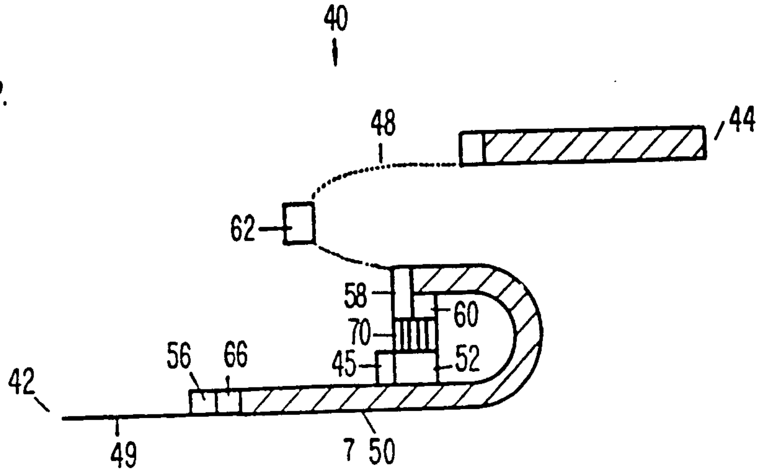


FIG. 2f.

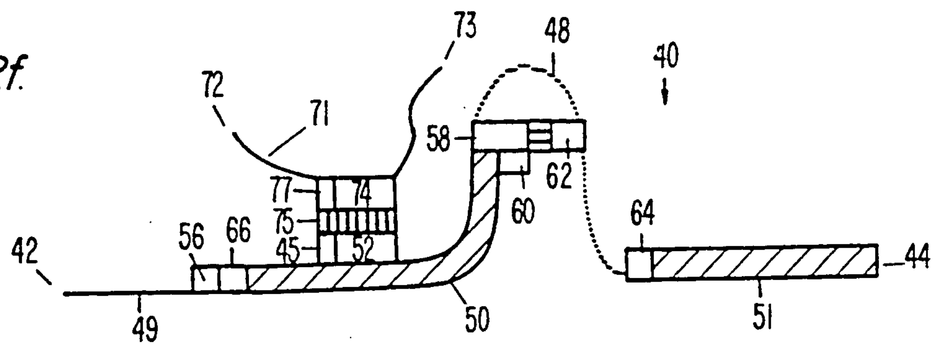
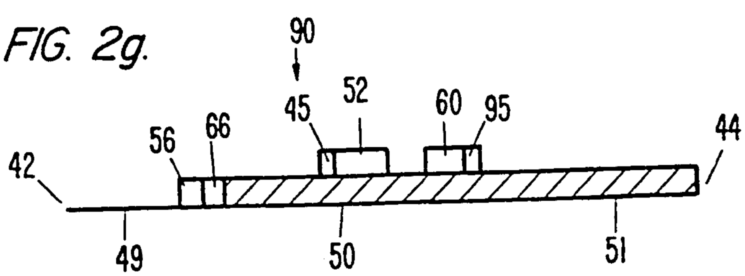
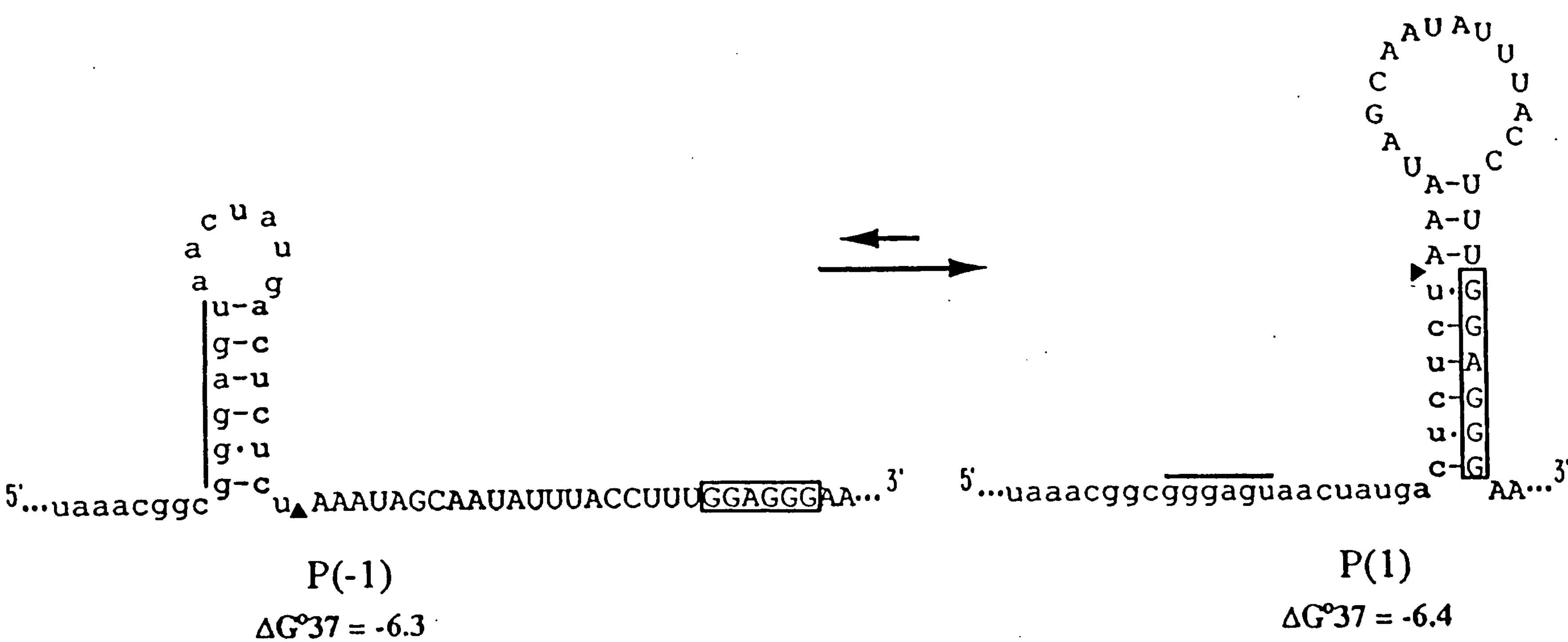


FIG. 2g.

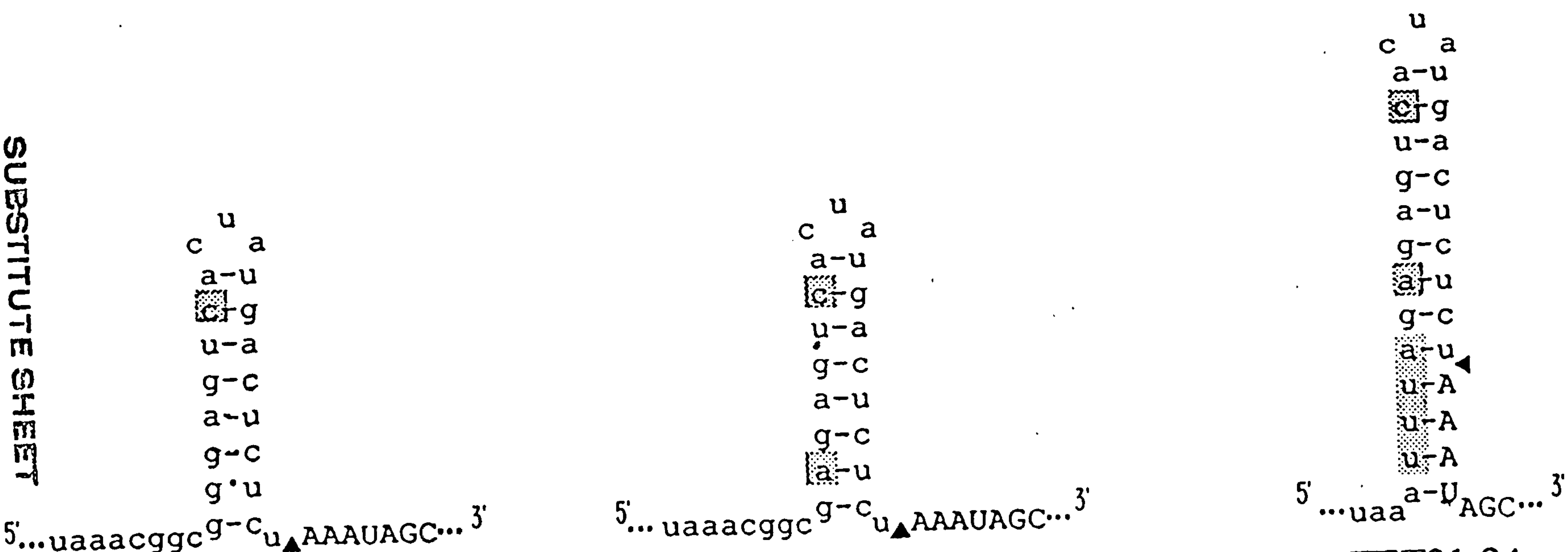




Alternative Conformations of 5' Splice Site RNA

pTETBLU

FIG. 3a.



pTET14 $\Delta G°37 = -10.2$

pTET1419 $\Delta G°37 = -11.2$

pTET21-24 $\Delta G°37 = -16.2$

Modified P(-1) Stem-loops

FIG. 3b.

FIG. 4.

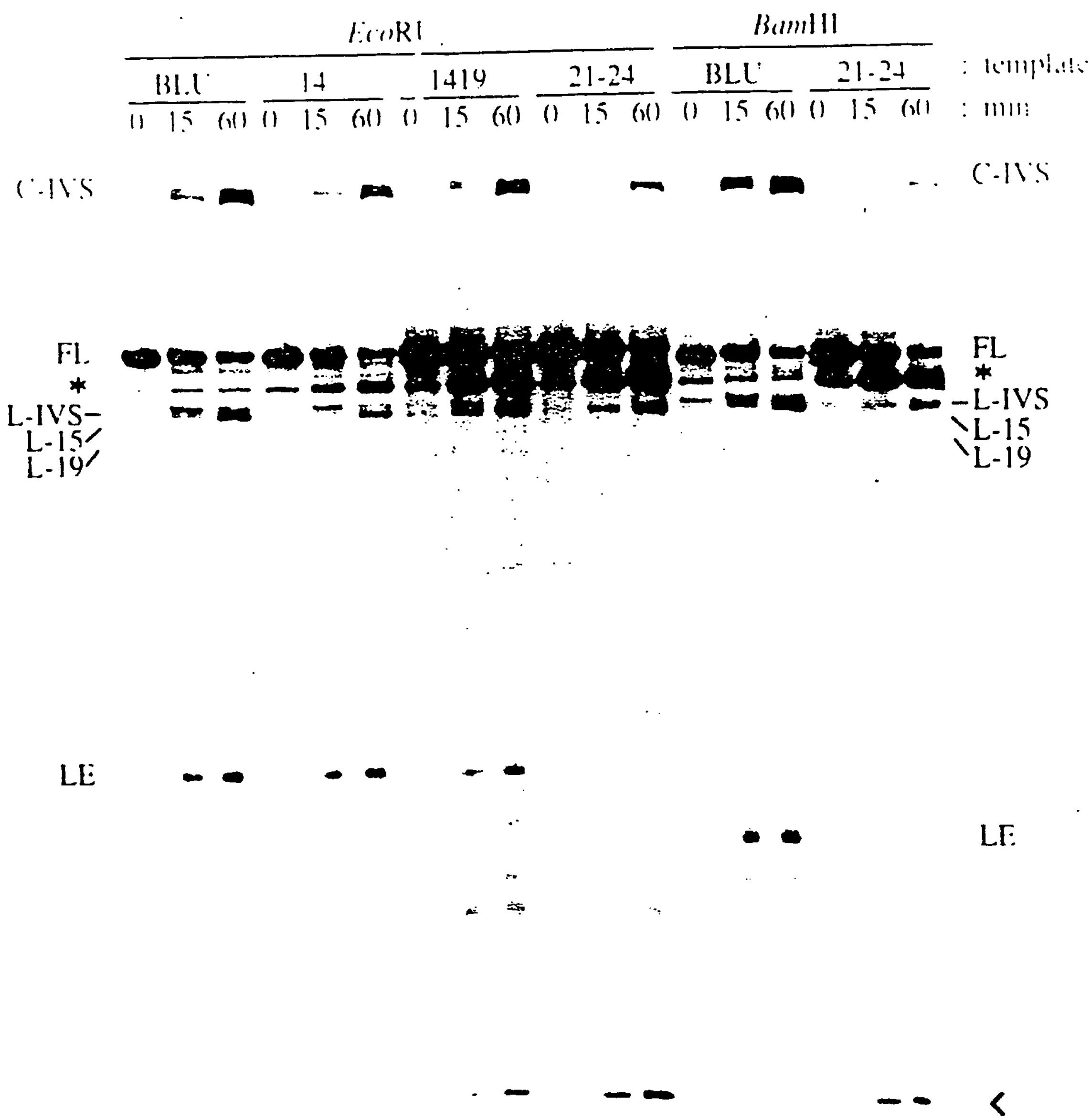

EcoRI
BamHI
: template
BLU
14
1419
21-24
BLU
21-24
: min
0 15 60 0 15 60 0 15 60 0 15 60 0 15 60 0 15 60
C-IVS
C-IVS
FL
*
L-IVS
L-15
L-19
FL
*
L-IVS
L-15
L-19
LE
LE

FIG. 5.

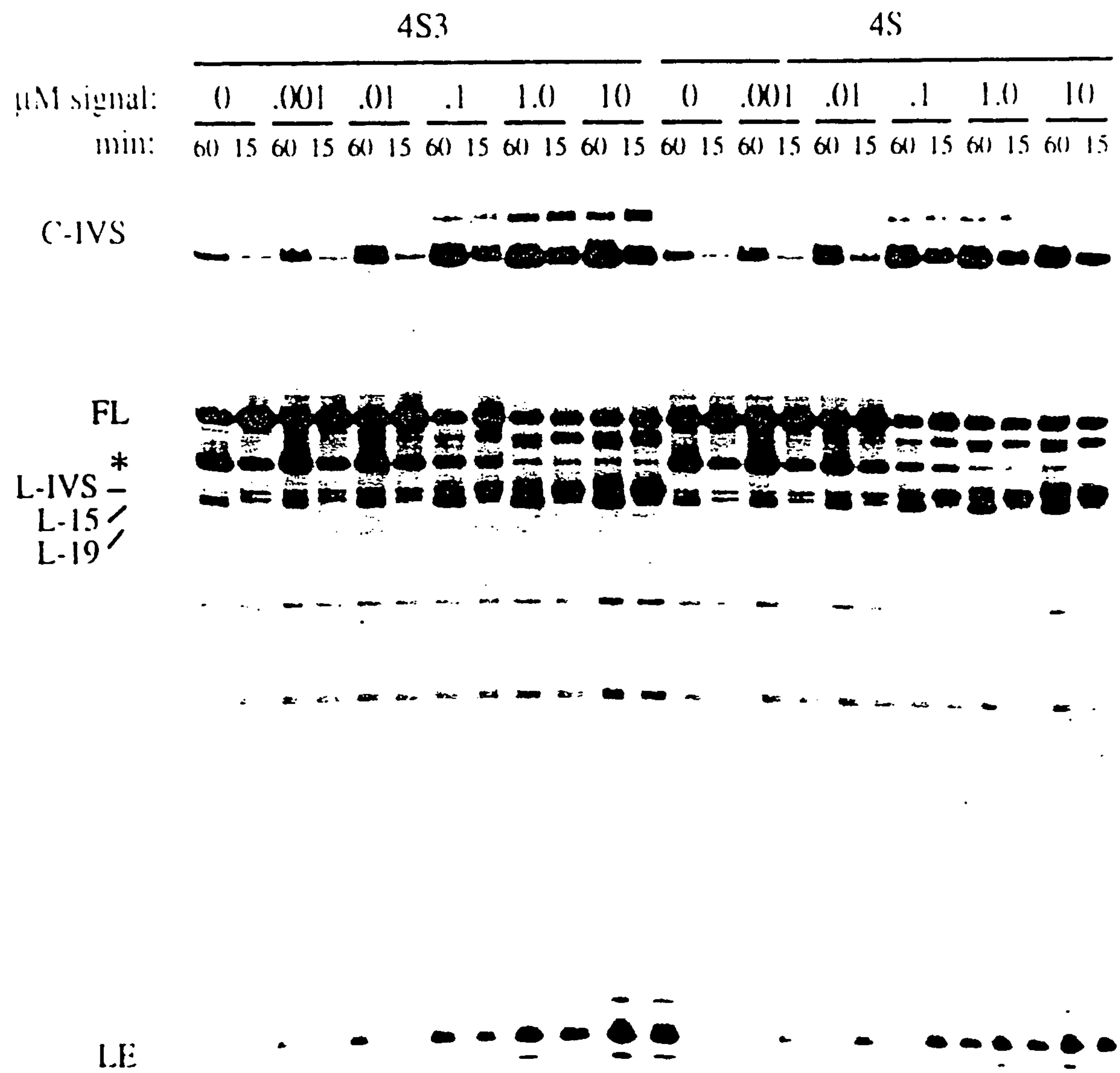
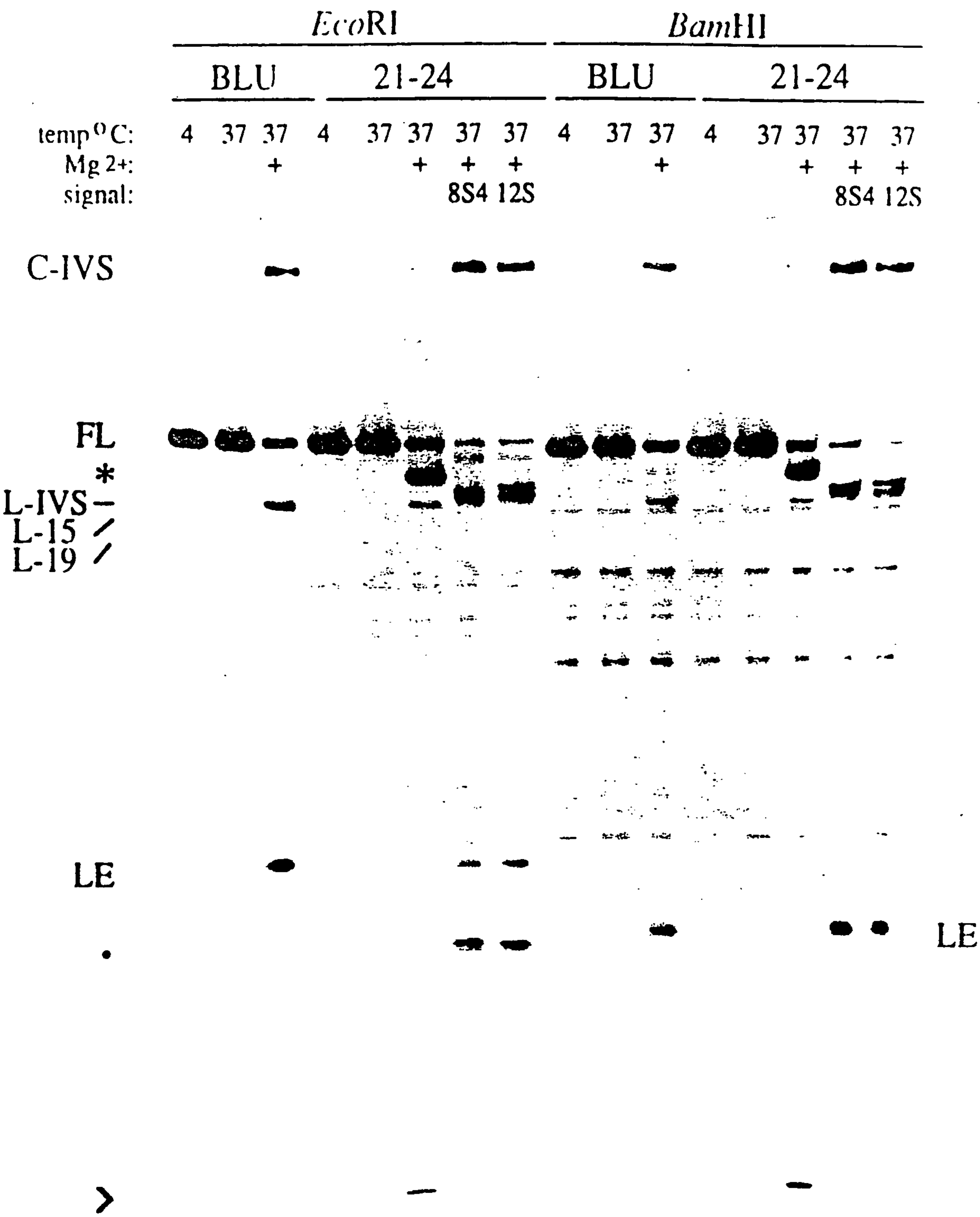

4S3
4S
μM signal: 0 .001 .01 .1 1.0 10 0 .001 .01 .1 1.0 10
min: 60 15 60 15 60 15 60 15 60 15 60 15 60 15 60 15 60 15 60 15 60 15 60 15
C-IVS
FL
*
L-IVS
L-15
L-19
LE

FIG. 6.


EcoRI
BamHI
BLU
21-24
BLU
21-24
temp °C: 4 37 37 4 37 37 37 37 4 37 37 4 37 37 37 37
Mg 2+: + + + + + + + +
signal: 8S4 12S 8S4 12S
C-IVS
FL
*
L-IVS
L-15
L-19
LE
LE

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04240

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 9/22; C12P 19/34; C07H 15/12, 17/00; A01H 1/00, 5/00
US CL :435/91, 193, 194, 172.3, 320.1; 800/205; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91, 193, 194, 172.3, 320.1; 800/205; 536/27;
935/3, 4, 34, 35, 44, 45, 83, 111

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS,
search terms: RNA, splic?, self-splic?, strand, displac?, translat?, R(w)loop, duplex, hybrid?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemistry, Volume 30, issued 1991, S. A. Woodson et al., "Alternative Secondary Structures in the 5' Exon Affect Both Forward and Reverse Self-Splicing of the *Tetrahymena* Intervening Sequence RNA", pages 2042-2050, especially pages 2043-2045.	1-27
Y	Science, Volume 228, issued 10 May 1985, J. V. Price et al., "Coupling of *Tetrahymena* Ribosomal RNA Splicing to Beta-Galactosidase Expression in *Escherichia coli*", pages 719-722, especially pages 719-720.	1-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
29 JUNE 1993

Date of mailing of the international search report
14 JUL 1993

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Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 347, issued 25 October 1990, C. Mariani et al., "Induction of Male Sterility in Plants by a Chimaeric Ribonuclease Gene", pages 737-741, especially pages 738-739.	1-27
Y	EP, A1, 0,242,016 (Baulcombe et al.) 21 October 1987, pages 1-12, especially page 2.	1-27
Y	EP, A1, 0,307,841 (Pfitzner et al.) 22 March 1989, pages 1-20, especially pages 2 and 6-9.	1-27
Y	Proceedings of the National Academy of Sciences, Volume 83, issued May 1986, M. Kozak, "Influences of mRNA Secondary Structure on Initiation by Eukaryotic Ribosomes", pages 2850-2854, especially pages 2851-2852.	6, 7